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13. ABSTRACT ***(Maximum 200 Words)***

We are investigating the role of AP-1 in controlling breast cell growth and transformation. We propose to determine the role of the AP-1 family of transcription factors in mediating peptide growth factor-induced proliferation and oncogene-induced transformation of breast cells. Previous results demonstrated that AP-1 complexes are activated by peptide and steroid growth factors in normal and malignant breast cells, and that normal breast cells express higher levels of AP-1 protein and activity than do breast cancer cells. We also previously showed that normal and immortal cells are more dependent on AP-1 for their growth than are most breast cancer cells. Over the last year, we determined whether AP-1 activation is required to transduce growth factor-induced signals in breast cancer cells. To perform these studies, we isolated MCF7 and MDA MB 435 clones that express a dominant-negative cJun mutant (TAM-67) under the control of an inducible promoter. These studies demonstrated that MCF7 cells, but not MDA MB 435 cells, depend on AP-1 for growth in serum. We also present results showing that inhibition of AP-1 completely blocked MCF7 proliferation induced by IGF-1 and EGF, yet only partially inhibited growth induced by estrogen. These results demonstrated that the mitogenic pathways in MCF7 cells activated by serum, estrogen, IGF-1, and EGF depend on AP-1 to transduce a proliferative signal, and that estrogen partially overcomes the growth suppressive effect of AP-1 blockade. These results suggest that AP-1 is a promising target of future cancer therapeutic and preventive agents since blocking this critical transcription factor suppresses proliferation induced by multiple growth factors.

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INTRODUCTION

During the final year of the funding period we have continued to investigate the affect of AP-1 blockade on the growth of breast cancer cells and have determined the mechanisms by which AP-1 blockade inhibits the growth of breast cells. By using our inducible MCF7-Tet-Off TAM67 cell lines, we demonstrated that TAM67 inhibits basal AP-1 activity and AP-1 activity stimulated by several different growth factors. We have also discovered that AP-1 activation is required to transduce growth factor-induced mitogenic signals in breast cells. These studies showed that inhibition of AP-1 completely blocked proliferation of MCF7 cells in response to the serum and peptide growth factors (IGF-1, EGF, heregulin-ß, and FGF). These results demonstrate that the mitogenic pathways activated by serum, IGF-1, EGF, heregulin-ß, and FGF depend on AP-1 to transduce a proliferative signal. We then determined the mechanisms by which TAM67 inhibits breast cancer cell growth. These studies showed that AP-1 blockade induced by the expression of TAM67 causes a G1 cell cycle arrest. In the absence of serum, TAM67 also causes apoptotic cell death. In addition, we have produced the TAM67 retroviral and adenoviral constructs. We have used these reagents to investigate the role of AP-1 transcriptional activity in oncogene-induced transformation of breast cells. However, over the last year, we have not been able to obtain high level of expression of TAM67 using these viral vectors. Therefore, we have proposed to begin a new project in which we will express the AP-1 inhibitor in transgenic mice to investigate the effect of AP-1 blockade on mammary gland development and breast cancer development in vivo. These studies have been proposed in a new DOD Idea grant proposal.

These studies have demonstrated that AP-1 is a critical regulator of breast cell growth. The results from these studies will provide the foundation for our ongoing efforts to develop agents that interfere with AP-1 signaling pathways. Such agents will likely be useful chemopreventive agents to block breast carcinogenesis.

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BACKGROUND

Breast cancer is the most common malignancy in women, and the leading cause of death for women between the ages of 40 and 55 in this country (1). Even with aggressive mammographic screening, adjuvant chemotherapy, and intensive therapy for existing cancer, many of the women who develop breast cancer will die from it. Thus, more effective prevention strategies and treatments are urgently needed.

Unfortunately, little is known about the specific molecular events, which cause the progressive transformation of human breast epithelial cells to malignant breast cancer. Studies of model systems of cancer have revealed that multiple steps are involved in carcinogenesis, including tumor "initiation" and "promotion" events (2). Mutations and deletions within tumor suppressor genes may represent the molecular equivalent of breast cancer "initiation" events (3,4). However, the molecular mechanism of breast tumor "promotion" is poorly defined. In model systems (5), classic tumor promoters induce the proliferation of initiated cells, leading to the progressive outgrowth of fully malignant cells. Such tumor promoters typically activate signal transduction pathways to stimulate cellular proliferation. In human breast cells, the overproduction of growth factors, or aberrant stimulation of growth factor receptors, may be responsible for the promotional phase of breast carcinogenesis. Growth factors important for mammary epithelial cells, such as estrogen, EGF, TGF-a, and the IGFs, may all represent tumor promoters of human breast cancer. Thus, drugs which inhibit the ability of estrogen to activate the estrogen receptor (tamoxifen and other antiestrogens) are used to treat breast cancer. Other drugs which block growth factor receptors, such as antibodies specific for the epidermal growth factor receptor and the Her2/neu receptor, have been shown to inhibit breast cancer cell proliferation (9-11), and are now being tested in clinical trials for the treatment of breast cancer. However, inhibition of individual signal transduction pathways may ultimately be ineffective, since multiple different signal transduction pathways can stimulate breast cell proliferation. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where multiple mitogenic signals converge. Since transcription factors, the nuclear proteins that control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction pathways, they may be ideal targets for new therapeutic agents.

A key family of transcription factors that transduces multiple mitogenic signals is the AP-1 family. These transcription factors are complexes of DNA-binding proteins made up of dimers of Jun and Fos proteins, which bind DNA at specific AP-1 sites and regulate the transcription of AP-1-dependent genes. AP-1 transcription factors are expressed in most cell types, and are activated by specific kinases, such as the mitogen-activated and stress-activated kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to UV light, oxidative stress, tumor promoters such as TPA, or oncogene overexpression or activation (reviewed in 6).

Thus, AP-1 is a central component of many signal transduction pathways in a variety of cell types.

Previous studies showed that the AP-1 transcription factor family is critical for growth factor induced proliferation of fibroblasts (12,13). In addition, we (14,15) and others (16) have shown that AP-1 is also critical for oncogene-induced transformation of fibroblasts. Specifically, we have demonstrated that AP-1 is critical for the cotransformation of primary rat embryo cells by *ras+jun*, *ras+fos*, or *ras+SV40 T antigen* (14), while others have shown that AP-1 is critical for the transformation of NIH3T3 cells by single oncogenes such as *ras*, *raf*, *abl*, and *mos* (16). Thus, AP-1 is a central regulator of transformation as well as mitogenic signaling.

While the role of AP-1 has been extensively studied in fibroblasts, relatively few studies of the function of AP-1 have been performed in epithelial cells. Thus the exact role of this transcription factor family in controlling the proliferation and transformation of epithelial cells is not known. Previous studies from our lab and others have demonstrated that the Jun and Fos family members are expressed in human breast cancer cells, and are activated by a variety of important growth factors for these cells, such as EGF, TGFa, and the IGFs. Other studies have also suggested that hormones such as estrogens and retinoids can modulate AP-1 transcriptional activity in breast cells. More recent studies suggest that ER and AP-1 interact to regulate the expression of certain estrogen and/or tamoxifen regulated genes (17). AP-1 complexes may be involved in regulating transcription of the ER gene as well (18). These results suggest that the AP-1 complex may be involved in controlling proliferation of human breast cells. However, definitive studies demonstrating that AP-1 is critical for either breast epithelial cell proliferation or transformation have not been performed.

To address these questions, we have used the 184 series of normal human mammary epithelial cells (HMECs) isolated and characterized by Dr. Martha Stampfer (19). These cells were originally isolated from reduction mammoplasties of patients and have a normal karyotype, EGF receptors, and specific cytokeratins, suggesting that they are derived from the basal epithelial cells of the normal breast. These HMECs are primary cells, which will senesce after 15-20 passages. However, by exposing these primary HMECs to the carcinogen benzo(a)pyrene, Stampfer *et al.* (19) have established multiple immortalized lines of HMECs (the 184A1 and 184B5 lines). We are studying these carcinogen-immortalized cells as well as the spontaneously immortalized HMEC line, MCF10A, derived from breast tissue obtained from a patient with multiple fibrocystic nodules (20). This cell line expresses cytokeratins and epithelial mucins consistent with a breast epithelial origin, and has cytologic characteristics of breast luminal ductal cells (21). None of the immortal cells are fully transformed since they are not able to grow in an anchorage-independent fashion, or form tumors in nude mice. Recent reports have demonstrated that these immortalized human mammary epithelial cells can be transformed by specific oncogenes such as activated *ras* (22, 23) or *erbB2* genes (24), or by overexpression of *c-myc* or *SV40 T* genes (16). In particular, MCF10A cells can be transformed by an activated *ras* gene (23), while 184B5 cells can

be fully transformed by activated *ras* genes (22), or by overexpression of c-*erbB2* (24). Many of these oncogenes are known to activate AP-1 in fibroblasts, though whether these oncogenes also activate AP-1 in breast epithelial cells is not yet known. If AP-1 is involved in regulating these processes, it might therefore serve as a target for the prevention or treatment of breast cancer. To determine the role of AP-1 in controlling breast cell growth and transformation, we proposed to test the following hypotheses:

1. Human breast epithelial cells at different stages in the carcinogenesis pathway express different levels of the AP-1 transcription factor.
2. Breast epithelial cells at these different stages have different requirements for AP-1 for their growth.
3. AP-1 transcription factor activity is necessary for *in vitro* transformation of human breast epithelial cells.

In our previous reports we demonstrated that AP-1 transcription factor expression and transcriptional activity is high in normal mammary epithelial cells and is progressively reduced as breast cells proceed towards malignancy. These studies were originally proposed in **Specific Aim 1**. We also previously reported data demonstrating that the growth of normal and immortal cells is suppressed by AP-1 blockade. These studies, proposed in **Specific Aim 2** of the original grant proposal, demonstrated that normal, immortal and some cancer cells depend on AP-1 to transduce mitogenic signals, and that normal cells are more sensitive to the AP-1 blockade than are breast cancer cells. Our results also suggest that mitogenesis induced by peptide growth factors, such as serum, EGF, IGF-1, is critically dependent on AP-1, while growth induced by estrogen is likely mediated partly through an AP-1-independent signal transduction pathway. Studies described in last year's annual report demonstrate that the mitogenic pathways in MCF-7 breast cancer cells activated by heregulin-ß, FGF, also depend on AP-1 to transduce a proliferative signal. These results are under review now at Cancer Research (manuscript included in appendix). We now report the results from our experiments performed over the previous and final year of this project. We have demonstrated that TAM67 inhibits breast cell growth by blocking the cell cycle. TAM67 induces a G1 cell cycle arrest. In addition, in the absence of serum, blockade of AP-1 by TAM67 induces apoptosis. We have also investigated the mechanism of this cell cycle block and have found that it is due to Rb hypophosphorylation induced by a increase in CDK inhibitors. To investigate whether AP-1 blockade suppresses the transformation of breast cells as described in **Specific Aim 3**, we produced a TAM67 retrovirus and an adenovirus expressing TAM67. Unfortunately, these reagents never expressed sufficient TAM67 in the breast cells to successfully complete this aim. Therefore, we have abandoned these *in vitro* studies, and we now plan to address this question using an *in vivo* model. We plan to prepare transgenic mice that express the TAM67 inhibitor in the mammary gland. In the future we will mate these TAM67 mice with transgenic mice that develop breast cancer to determine whether AP-1 blockade can prevent mammary tumor formation in vivo. This proposed series of experiments was submitted as an Idea Grant Proposal to the DOD this year.

EXPERIMENTAL METHODS AND PROCEDURES

Primary Cell Cultures and Cell Lines:

Human mammary epithelial cells and cell lines used in these studies (Table 1) include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9-10); normal 184 cells (15); 184A1 and 184B5, nontumorigenic immortal cell lines derived from benzo(a)pyrene-treated 184 cells (19); MCF10A (from Dr. J. Russo), a nontumorigenic spontaneously immortalized HMEC cell line; MCF10AneoT (from Dr. J. Russo, Fox Chase Cancer Center, Philadelphia, PA), a transformed cell line derived from MCF10A transfected by c-Ha-ras; MCF7 WT (wild-type), a human breast adenocarcinoma cell line; MCF7 Adria, a doxorubicin (Adriamycin)-resistant subclone of MCF7 WT (from Dr. K. Cowan, National Cancer Institute, Bethesda, MD). Cells were grown in the following culture media: MEGM (Clonetics, San Diego, CA) for normal HMECs 184, 184A1, and 184B5 (19,25); DME/F-12 with 5% horse serum and supplements as described (20, 23) for MCF10A and MCF10AneoT [with 400 μg/ml Geneticin (G418, Life Technologies, Inc., Gaithersburg, MD); and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for MCF7 and MDA MB 435.

Plasmids:

To develop the pLPCX-TAM67 retroviral construct, the TAM67 fragment (0.6 kb) which is tagged with FLAG was released from pCDNA(-)3.1-FLAG-TAM67 with HindIII/XhoI. The fragment was re-ligated to the pLPCX (Clontech) retroviral vector. To produce the pCA14-TAM67 adenoviral construct, the TAM67 fragment (0.66 kb) which is tagged with FLAG was released from pCDNA(-)3.1-FLAG-TAM67 with EcoRI/HindIII. The fragment was then cloned into the pCA14 shuttle vector. This vector is being used to produce the TAM67 adenovirus.

Transfection of Breast Cells:

184, clone 91, 184B5, MDA MB 231, MCF7, and T47-D breast cells were transfected using Fugene 6 reagent (Boehringer Mannheim) while MCF10A and MDA MB 435 breast cells were transfected using the LT-1 transfection reagent (PanVera Corp.) according to manufacturer's recommendations.

Western Blot Analysis:

Equal amounts of total cellular protein extract were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). Primary antibody used was rabbit anti-cJun Ab-1 from Oncogene Science (Cambridge, MA). Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

Luciferase Assay to Measure AP-1 Activity:

AP-1 transcriptional activating activity in cells was measured using the Dual-Luciferase™ Reporter Assay (Promega) as previously described (13). The cells were

co-transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter which contains a single AP-1 binding site (TGAG/CTCA) between nucleotides –73 and –60 and pRL-TK. To determine the AP-1 activity stimulated by growth factors, the cells were treated with EGF (100 ng/ml), IGF-1 (100 ng/ml), herogulin-β1 (10 ng/ml) or b-FGF (10 ng/ml), respectively for 6 hours before harvest. Transfected cells were lysed 36 hours after transfection and luciferase activity was measured with equal amounts of cell extract and normalized with the Renilla activity.

Cell Growth Assays:

Cell proliferation assay of stably transfected Tet-On and Tet-Off cell lines

The CellTiter 96^{TM} AQueous Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, Madison, WI) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. 1000 to 2000 cells were seeded in a 96 well plate and doxycycline was added (MDA MB435 rtTA-vector or -TAM67 lines) or removed (MCF7 tTA-vector or -TAM67 lines) was added the next day and replaced every other day. A solution containing a 20:1 ratio of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium) and PMS (phenazine methosulfate) was added to the cells for 2 hours at 37° C and absorption at 495 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Cell proliferation assay of breast cells treated with specific growth factors:

The MTS assay described above was used to measure breast cancer cell growth after stimulation with specific growth factors, including heregulin-ß1 (0 to 10 ng/ml), b-FGF (0 to 10ng/ml. The cells were seeded in 24 well plates in full medium with doxycline (MCF7 cells) or without doxycycline (MDAMB435 cells). The cells were allowed to attach overnight, and then were washed and cultured in serum free medium (and in the case of estrogen treated cells, estrogen- and phenol red-free medium) for 48 hours. The media was then changed to doxycline-free media (MCF7 cells) or doxcycline-containing media (MDAMB435 cells), to induce TAM-67 protein, with different levels of the specific growth factors. The cells were then cultured at 37° C for 1 to 7 days. Cells were harvested every other day and the MTS assay was done as described above to measure proliferation. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

^{3}H-Thymidine Incorporation Assay:

3 x 10^4 cells were seeded in a 24-well plate and then were starved in medium without any growth factors to synchronize the cells. The cells were then labeled with ^{3}H-thymidine (2uCi/ml) for 1 hour followed by incubated with 5% TCA at 4°C for 30 minutes. The cells were then lysed by addition of 0.1 N NaOH. 3H-thymidine uptake was measured by mixing the cell lysates with scintillation fluid and counting the ^{3}H cpm in a scintillation counter. Each data point was performed in sextuplet, and the results were reported as cpm +/- standard error. All results were normalized to cell numbers.

Flow Cytometry Assay:
MCF-7 Tet-Off TAM67 Clone #62 cells were kept in –Dox and +Dox media to induce or block the expression of TAM67. 24 hours before harvest, the medium is changed to serum-free medium to synchronize the cells. 2x 10^6 cells were harvested at time point 0h, 6h, 12h, 24h, 48h, and fixed in 95% ethanol for 30 min in room temperature, and then stored in 4°C until ready to stain. For propidium iodide staining, the cells were pelleted and resuspended in 1 X PBS. 1 ml of 50 ug/ml of PI was used to stain the cells. Stained cells were analyzed with EPICS XL-MCL flow cytometer (Coulter Co.). Histograms were then analyzed for cell cycle compartments.

Apoptosis Assay:
MCF-7 Tet-Off TAM67 Clone #62 cells were kept in –Dox and +Dox media to induce or block the expression of TAM67. For TUNEL assay, the cells were trypinized and pelleted. These samples were then embedded on agar, cut and mounted on slides and processed for TUNEL assay as previously described. For cell death ELISA assay, 1 x10^4 cells were seeded in 96 well plate overnight. This apoptosis assay measures cytoplasmic DNA fragments and was performed according to the protocol of Cell Death ELISA Kit (Roche). Each sample was performed in triplet and the results expressed at mean +/- standard error from at least two independent experiments.

Retrovirus Preparation and Infection:
Phoenix ampho cells were maintained in Dulbecco's Modified Eagle Medium supplied with 10 % of fetal bovine serum, 1 % of penicillin/streptomycin and 1 % of L-glutamine. 2.0 x 10^6 cells were seeded in a 60-mm dish 18-24 hours prior to transfection. pLPCX-TAM67 and control plasmids were transfected with Fugene 6 Regent (Roche) according to manufacture's protocol. Chloroquine was used to increase the yield of retrovirus. 48 hours post-transfection, the retroviral supernatants were harvested and filtered through a 0.45 um filter, then kept in –70°C freezer for future use. The titers of the retrovirus were determined by infecting NIH3T3 cells and using limiting dilution according to the protocol from Clontech. For infection with retrovirus, 5 x10^5 cells were plated into 10 cm dish 12-18 hours prior to infection, the cells were then treated for 6 hours or overnight with infection cocktail containing equal amounts of retroviral supernatant and growth medium and 4 ug/ml polybrene. After infection, the medium was changed to normal growth medium and the cells were incubated for another 36 to 48 hours. pLEGFP-N1 retroviral supernatant was also used as control.

Soft Agar Cloning :
Tp prepare plates for soft agar cloning, SeaPlaque Agarose (low gelling) was added to the stock solution to produce a 3.5 % stock solution made with PBS. Pre-warmed media was added to get a 0.7 % agarose. For the lower feeder layer, 1.5ml of 0.7 % of agarose was added to 6-well plate and then allowed to solidify by incubating the plate at 4°C for 20 to 30 min. The cells were trypsinized and a single cell suspension was made. A 0.35 % solution of agarose was prepared by adding five thousand cells to 4

ml of agarose. 4ml of cell/agarose were plated into the 6-well plate with solidified bottom layer. The plate was then placed into 4°C refrigerator for 1 to 2 hours to solidify the upper layer and then transferred to 37°C incubator. The clones were allowed to grow for 14 days and then were counted.

RESULTS

AP-1 Expression and Activity in Breast Cells:

In **Specific Aim 1** we proposed to determine whether changes in AP-1 expression or activity occur as breast cells progress through different stages of carcinogenesis. Breast cells used in this study are listed in Table 1. We have previously shown that normal human mammary epithelial cells have high basal AP-1 activity, immortal breast cells have an intermediate level of basal AP-1 activity, and breast cancer cells have low basal AP-1 activity. We described these results in the 1996-97 annual report and in a 1997 *Cancer Research* publication (Smith *et al.* 28).

Table 1: Breast cells used in this study.

Cells	Name	Source	Phenotype
Normal HMECs:	HMEC-91	Clonetics	Senescent, anchorage-dependent
	184	M. Stampfer	
Immortal HMECs:	184B5	M. Stampfer	Immortal, anchorage dependent
	MCF10A	A. Russo	
Breast Cancer cell lines:	MCF7 WT	K. Cowan	Cancer cells, anchorage-independent and tumorigenic
	T47-D	ATCC	
	MDA MB 231	ATCC	
	MDA MB 435	ATCC	

In **Specific Aim 2** we proposed to determine whether the growth of breast cells at different stages of tumorigenesis are differentially affected by inhibiting AP-1 activity. We have investigated the affect of AP-1 blockade on the growth of normal, immortal, and fully malignant breast cells. These studies have demonstrated that the growth of normal and immortal and some malignant cells is suppressed by AP-1 blockade, while the growth of some other breast cancer cells is not suppressed. These results were described in the 1998-99 annual reports and presented as abstract at the 2000 AACR Annual Meeting in San Francisco and in the recently published manuscript in *Oncogene* by Ludes-Meyers *et al.* (Ludes-Meyers *et al.* 29).

Tet-On And Tet-Off Inducible Breast Cancer Cells Expressing TAM-67:

We previously described the isolation of breast cancer cell lines that express TAM-67 under the control of Tet-inducible or Tet-repressible promoters. For these experiments described in this report, we used: MCF7-Tet-Off TAM-67 cells: These cells do not express TAM-67 when the cells are grown in doxycycline (1ug/ml), but do express TAM-67 protein when grown in the absence of doxycycline.

Effect of Inhibiting AP-1 Transactivating Activity on Proliferation Induced by Specific Growth Factors:

In our last year's annual report, we described that AP-1 blockade induced by overexpression of TAM67 inhibited MCF-7 cell growth. TAM67 also inhibited MCF-7 growth induced by EGF, IGF-1, and partially inhibited the growth induced by estrogen. Over the last year, we have investigated the effect of blocking AP-1 on mitogenesis induced by other growth factors. In these experiments, we found that TAM67 inhibited basal AP-1 activity and AP-1 activity stimulated by different growth factors (IGF-1, EGF, heregulin-β, and FGF) (Fig 1). MCF7 Tet-Off TAM-67 cells (Clone #62), or MCF7 Tet-Off vector cells (Vector Clone #1) were treated with different concentrations of heregulin-β (0, 0.1, 1.0, 10.0 ng/ml), or FGF (0, 1.0, 10.0, 100.0 ng/ml) in the presence and absence of doxycycline. When the TAM-67 MCF7-Tet-Off clone was grown in the presence of doxycycline (no TAM-67 expressed), these MCF-7 clones proliferated normally in response to serum stimulation. However, when doxycycline was withdrawn, TAM-67 was expressed, and growth factor-induced proliferation was inhibited (Fig 2, 3). These studies are described in the manuscript by Liu *et al.* included in the appendix. This manuscript has recently been submitted for publication to Cancer Research and are now under review.

Mechanism by Which TAM67 Inhibits Breast Cancer Cell Growth:

We have previously demonstrated that AP-1 blockade inhibits normal and malignant breast cell growth. We predicted that AP-1 blockade causes either cell cycle block or apoptosis. To determine whether AP-1 blockade suppresses DNA synthesis, we used the MCF-7-Tet-Off-TAM67 cells under un-inducible and inducible conditions to measure ^{3}H-Thymidine uptake. The results demonstrated that the expression of TAM67 inhibits the DNA uptake of MCF-7 cells (Fig. 4). Then we performed flow cytometry assay under same conditions. The flow cytometry assay results showed that when Dox was induced, there were fewer cells in S phase and more cells in G0/G1 phase compared to cells were cultured in Dox-containing medium (Fig. 5). The results from ^{3}H-Thymidine uptake and flow cytometry are consistent, which demonstrate that the AP-1 blockade induced by the expression of TAM67 causes the G1 cell cycle arrest of MCF-7 breast cancer cells.

We next investigated whether AP-1 blockade induced apoptosis. Results from Cell Death ELISA Assay demonstrated that the expression of TAM67 did not induce apoptosis in MCF-7 cells when the cells were cultured in normal full medium (Fig 6). TUNEL results also confirmed these results. However, when the cells were cultured in serum-free medium, TAM67 caused apoptosis (Fig. 6). These studies demonstrated that AP-1 blockade induced by expression of TAM67 inhibit MCF-7 breast cancer cell growth mainly by inducing G1 cell cycle arrest. The results of experiments investigating the mechanism by which TAM67 inhibits breast cell growth are included in a manuscript in preparation entitled "AP-1 Blockade Inhibits Breast Cancer Growth by

Inducing a Cell Cycle Block".

Suppression of Oncogene-induced Transformation by AP-1 Blockade:

In **Specific Aim 3** we proposed to determine whether inhibition of AP-1 activity prevents the *in vitro* transformation of immortalized breast cells. We have previously successfully transfected oncogenes, such as c-Ha-ras or c-erbB2, into immortal 184B5 and MCF10A cells and transformed these cells into cells that exhibit anchorage-independent growth. We are now using two different approaches to investigate whether TAM-67 blocks oncogene-induced transformation:

1). Over the last year we have attempted to reverse the phenotype of already transformed cells. For these experiments, we constructed pLPCX-TAM67 retroviral vector. We transfected the vector into Pheonix ampco package cells and harvested viral supernatant. We infected the Ras- and erbB2- transfected 184B5 and MCF10A cells with the virus. We will then measure the effect of AP-1 blockade on the anchorage independent growth of these cells in soft agar.
2). As an alternate strategy, we constructed an adenovirus expressing TAM67. We used this adenovirus to infect oncogene-transfected immortal breast cells.

We have attempted to suppress breast cell transformation using each of these two viruses that carry TAM67 (pLPCX-TAM67 and Adeno-TAM67). Unfortunately, we have not obtained high levels of expression with either of these two viral vectors.

Unfortunately, over the last year, despite aggressive attempts to get high expression of TAM67 protein in the 184B5 and MCF10A cells, we were unable to obtain viruses that would produce high TAM67 expression. This may be due to the growth suppressive effects of TAM67. Any virus that expresses TAM67 to high levels will likely stop the host cell from growing. Thus, we were unable to suppress oncogene-induced breast cell transformation using this in vitro approach.

These studies complete the proposed work. To continue to try to determine whether AP-1 blockade suppresses breast cell transformation, we have recently proposed experiments to block oncogene-induced transformation of breast cells *in vivo*. Because of the problems that we have encountered trying to block oncogene-induced transformation in vitro, these new studies will be done *in vivo* using transgenic mice. Therefore, we have proposed to make a transgenic mouse that expresses the TAM67 gene under a tissue-specific inducible promoter using Tet repsonsive-TAM67 mice crossed with MMTV-Tet Activator mice. Once prepared these mice will then be mated to MMTV-erbB2 and MMTV-ras mice to determine whether AP-1 blockade can inhibit oncogene-induced mammary tumor formation *in vivo*. These studies were described in a DOD Idea grant proposal submitted this year.

DISCUSSION

The data presented here, along with our previous data, demonstrate that AP-1 blockade induced by TAM-67 inhibits the growth of normal, immortal and some breast cancer cells (such as MCF7 cells), but that other breast cancer cells (such as MDA MB 435) are relatively resistant to AP-1 blockade. The present results also show that mitogenesis induced by individual peptide growth factors, such as IGF-1, EGF, heregulin-ß, and FGF, can be completely blocked by expression of TAM-67, while mitogenesis induced by estrogen is only partially inhibited by AP-1 blockade. These data suggest that peptide growth factor-induced mitogenesis is critically dependent on AP-1 in MCF-7 cells. The data also suggest that estrogen-induced mitogenesis is only partially dependent on AP-1 and that estrogen-induced signaling likely involved other AP-1 independent pathways. Our recent data also demonstrate that AP-1 blockade induced by expression of TAM67 causes growth inhibition by suppressing entry into S-phase of the cell cycle. In the absence of serum, expression of TAM67 also induces apoptosis.

Multiple growth factors have been shown to stimulate the proliferation or differentiation of normal HMECs and breast cancer cells (15, 29,30). EGF stimulates the growth of normal HMECs (15) as well as breast cancer cells (29,30), and heregulin has been found to modulate the growth and differentiation of immortal HMECs (31). Other hormones that affect the growth of breast cancer cells include estrogen (32,33) and insulin-like growth factors (IGF-1 and IGF-2) (34), which induce proliferation, and retinoids, which inhibit proliferation and induce differentiation (35,36). Previous studies from our own lab have demonstrated AP-1 complexes are activated by important growth factors for breast cells, such as EGF, and IGFs (28). These previous results suggested that the AP-1 complexes might be involved in controlling proliferation of human breast cells. The current data now demonstrated that peptide growth factors stimulate breast cancer cell proliferation through the AP-1 signaling pathway. Thus, the AP-1 transcription factor is a critical signaling molecule in normal breast cells and in some breast cancer cells (i.e. MCF-7), but apparently not in other breast cancer cells (i.e. MDA MB 435).

C-Jun is a major component of the AP-1 transcriptional complex. AP-1 plays a critical role in cell's proliferation and transformation. For example, homozygous jun-/- mouse embryos die after 12-14 days at mid-to-late gestation (37, 38). In addition, cellular transformation induced by many oncogenes requires c-jun (39). AP-1 functions by regulating AP-1 dependent downstream genes, or by interacting with transcriptional co-activators, such as JAB1 (Jun activation domain binding protein 1), or integrators, such as CBP or p300 (40, 41, 42). However, AP-1 activated genes whose products participate in the progression from G1 to S phase are yet to be identified. Here we showed data demonstrating that AP-1 blockade induced by expression of TAM67 delays S phase entry and causes G1 cell cycle arrest. We are currently investigating the

mechanism by which AP-1 blockade delays S phase entry.

Recently, it has been suggested that AP-1 components and their upstream kinases (mainly JNKs) may also be involved in apoptosis (43-45). Although much attention has been directed towards the possible role of AP-1 in the induction of apoptosis, it should be noted that AP-1 may play a totally opposite role, namely providing protective function in response to some stresses in some cell types (46-48). Thus, inhibition of AP-1 may induce apoptosis in some conditions. In our studies, AP-1 blockade induced by expression of TAM67 induces apoptosis in serum free medium, but not in the presence of serum.

We have previously demonstrated that normal human breast cells have high basal levels of AP-1 activity and that breast cancer cells express relatively low levels of AP-1 activity. We have also shown that AP-1 complexes are activated by peptide and steroid growth factors in normal and malignant breast cells. In this report, we now show that other peptide growth factors, including IGF-1, EGF, heregulin-β, and FGF, stimulate AP-1 activity in breast cancer cells. This growth factor-induced AP-1 activity can be suppressed by the expression of TAM67. The mitogenic pathways activated by serum and these growth factors depend on AP-1 to transduce proliferative signal, and that estrogen-induced growth is only partially inhibited by AP-1 blockade. AP-1 blockade induced by the expression of TAM67 inhibits breast cancer cell growth mainly by delaying S phase entry, and inducing G1 cell cycle arrest. In the future, we will determine whether AP-1 blockade inhibits breast cell growth *in vivo* and whether AP-1 blockade prevents oncogene-induced transformation of breast cells. These results suggest that AP-1 is a promising target of future cancer therapeutic and preventive agents since blocking this critical transcription factor suppresses proliferation induced by multiple growth factors.

FUTURE STUDIES

We have now completed the work proposed in this award. As described above, we are planning to continue studies of the role of AP-1 in transducing proliferative and transforming signals in breast cells using transgenic mouse models. These transgenic mouse studies have been submitted to the DOD as an Idea award proposal.

KEY RESEARCH ACCOMPLISHMENTS AND REPORTABLE OUTCOMES

Specific Aim 1: To determine whether changes in AP-1 expression or activity occur as HMECs progress through different stages of carcinogenesis.
We have completed this specific aim and have completed tasks for months 1-36 listed in the statement of work.

Accomplishments:

Our results demonstrated that Jun and Fos protein expression and AP-1 activity are high in normal human mammary epithelial cells, and are reduced as breast cells progress toward a more malignant phenotype

Reportable Outcomes:

The results of these studies were:

1. described in our first year annual report,
2. presented at the "Era of Hope" meeting in Washington, D.C., November, 1997,
3. published as an abstract in the Era of Hope meeting booklet, 1997
4. published in *Cancer Research* (Smith *et al.* , 1997) (28).

Specific Aim 2: To determine whether growth of HMECs at the different stages is differentially affected by inhibiting AP-1 activity.

The tasks for months 1-36 have been completed and summarized in our previous reports and in this report.

Accomplishments:

1. These studies demonstrate that normal and immortal human mammary epithelial cells require AP-1 for their growth.
2. We successfully established MCF7 Tet-Off-TAM67 and MDAMB435 Tet-On-TAM67 cell lines.
3. We discovered that breast cancer cells, which have relatively low basal AP-1 transcriptional activity, are less sensitive to AP-1 blockade. Of the breast cancer cells tested, MCF7 cells were the most sensitive to the growth suppressive effect of the AP-1 inhibitor.
4. We also found that AP-1 blockade completely inhibits proliferation of breast cancer cells in response to peptide growth factors (IGF-1, EGF, heregulin-ß, and FGF), but only partially inhibits estrogen-induced proliferation.
5. We determine the mechanisms by which TAM67 inhibits breast cancer cell growth. TAM67 mainly induces G1 cell arrest, and causes apoptosis in serum-free condition.

Reportable Outcomes:

These results were:

1. presented at the "*Era of Hope*" meeting in Washington, D.C., in November 1997 and published as an abstract in the meeting booklet.
2. presented at the San Antonio Breast Cancer Symposium in 1997 and 1998, and published as an abstract in *Breast Cancer Research and Treatment.*
3. presented at the *91st AACR* Annual Meeting, April, 2000, San Francisco as a poster and published as an abstract in Proceedings of the American Association for Cancer Research, 41:500,2000.

4. presented at the "*Era of Hope*" meeting in Atlanta, June 8-11, 2000 as a poster and published as an abstract in the meeting booklet
5. described in the manuscript published in *Oncogene* (Ludes-Meyers et al, 2001, 49).
6. described in the manuscript submitted to *Cancer Research* (Liu et al.)

Specific Aim 3: To determine whether inhibition of AP-1 activity can prevent the *in vitro* transformation of immortalized HMECs.
All tasks have been completed.

Accomplishments:

1. We have developed an *in vitro* transformation assay and have isolated oncogene-transformed HMECs and determined their transformed phenotype.
2. We have established several clones of 184B5 and MCF10A cells that stably express an activated erbB2 oncogene or an oncogenic ras protein. All of these clones exhibit the transformed phenotype of anchorage independent growth.
3. These transformed HMECs and breast cancer cells were used to determine whether inhibition of AP-1 transcriptional activity reverses the transformed phenotype of breast cells – we saw no inhibition of transformation, but did not achieve high expression of the TAM67 using the retroviruses or adenoviruses.
4. We developed TAM67 retroviral vector---pLPCX-TAM67
5. We developed TAM67 adenoviral vector – Adeno-TAM67

Reportable Outcomes:

1. We have derived oncogene-transformed 184B5 and MCF10A cell lines.
2. Constructed a pLPCX-TAM67 retrovirus.
3. Constructed a TAM67 adenovirus; however this virus did not express the TAM67 protein.

CONCLUSIONS

During the fourth year of the funding period, we investigated the effects of AP-1 blockade induced by TAM67 on breast cell growth induced by growth factors and determined the mechanisms by which TAM67 inhibits breast cancer cell growth. Our results demonstrated that expression of TAM67 inhibited basal AP-1 activity and AP-1 activity stimulated by growth factors, including EGF, IGF-1, heregulin-ß, FGF. Our data also demonstrated that AP-1 blockade inhibits MCF-7 cell growth stimulated by serum, EGF, IGF-1, heregulin-ß, FGF. These data suggest that peptide growth factor-induced mitogenesis is critically dependent on AP-1 in MCF-7 cells. In addition, we investigated the mechanisms by which AP-1 blockade induced by expression of TAM67 inhibits breast cell growth. The results demonstrated that TAM67 mainly induces G1 cell cycle arrest, and under serum-free condition, it causes apoptotic cell death to suppress MCF-7 cell growth. We also have developed TAM67 retrovirus and now making TAM67 expressing adenovirus which can be used to infected breast cancer cells and oncogene-transformed HMECs to investigate whether AP-1 blockade suppresses the transformed phenotype of oncogene-transformed human breast cells.

These studies have demonstrated an involvement of AP-1 transcription complexes in regulating human breast cancer cell proliferation through different signaling pathways. The results from these studies will provide the foundation for future efforts to develop agents that interfere with AP-1 signaling pathways. Such agents may be useful chemopreventive agents to block breast carcinogenesis.

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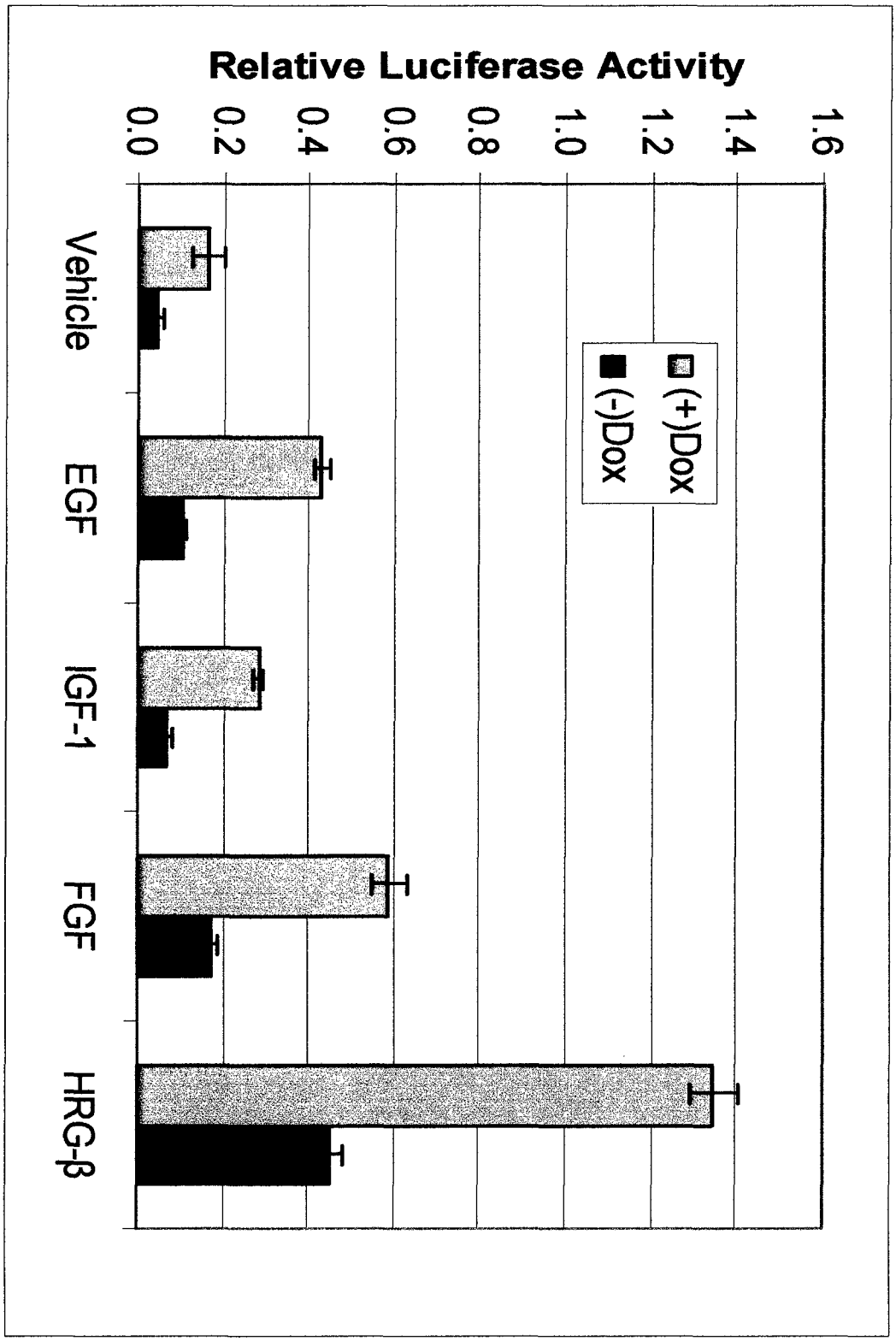
ABBREVIATIONS

AP-1	Activating Protein 1
ATCC	American Type Culture Collection
ATF	Activating Transcription Factor
bp	base pairs
CBP	Creb Binding Protein
cDNA	complementary Deoxyribonucleic Acid
CMV	Cytomegalovirus
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced Chemiluminesence
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
ERE	estrogen responsive element
FCS	Fetal Calf Serum
FGF	fibroblast growth factor
Ha-ras	Harvey-ras
HMEC	Human Mammary Epithelial Cells
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like growth factor binding protein
MAPK	mitogen activated protein kinase
MEGM	Mammary Epithelial Growth Medium
MEM	Modified Eagle Medium
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
neo	neomycin transferase
nm	nanometer
O.D.	Optical Density
PMS	phenazine methosulfate
rtTA	reverse tetracycline-controlled transactivator
SEM	Standard Error of the Mean
SV40	Simian Virus 40
tTA	tetracycline-controlled transactivator
Tet	Tetracycline
TetRE	Tetracycline Response Element
TGF	Transforming Growth Factor
TKR	tyrosine kinase receptor
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TRE	TPA responsive element
TUNEL	TdT (terminal deoxynucleotidyl transferase)-mediated dUTP-x nick end labeling
WT	Wild-type


Relative Luciferase Activity
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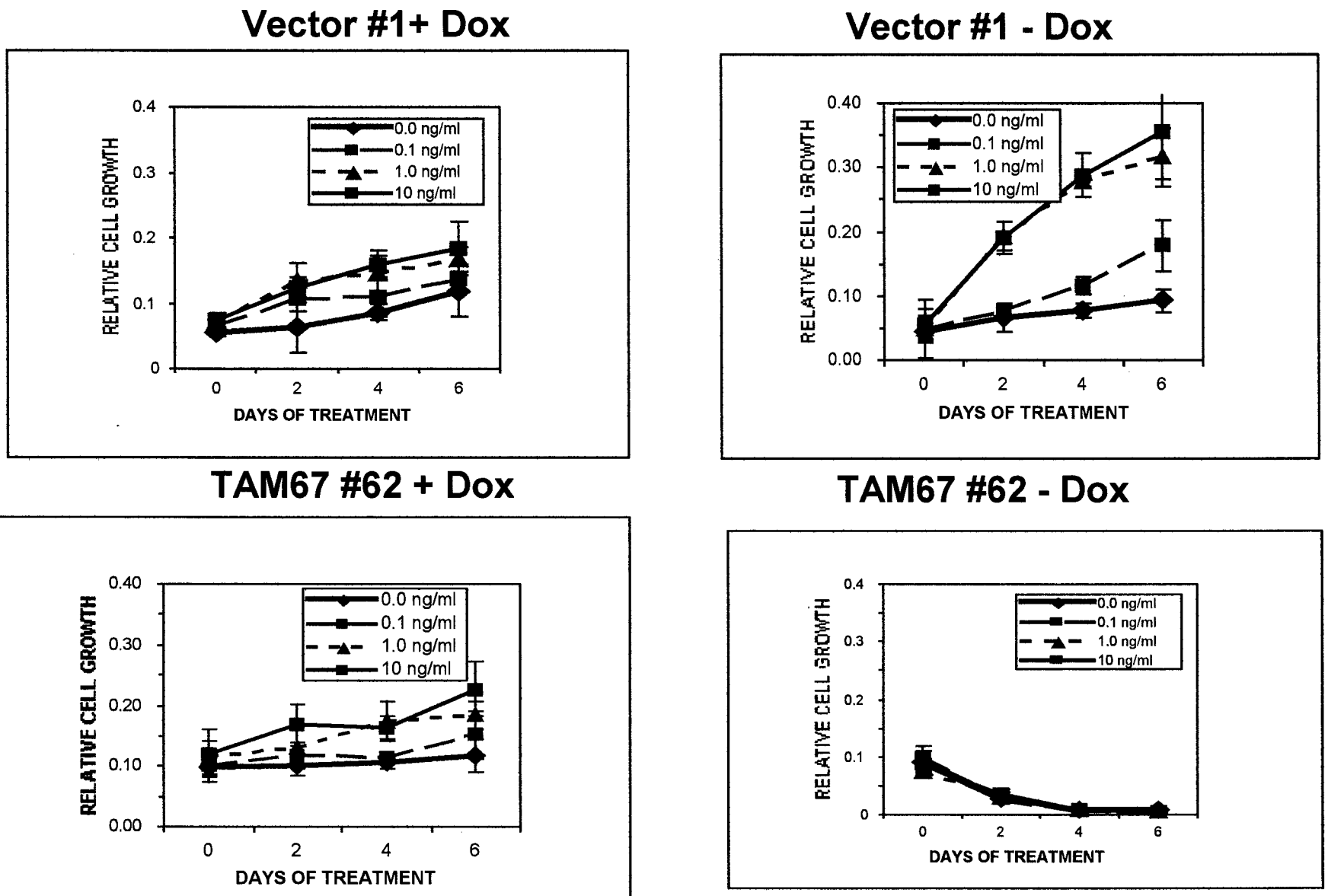
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Vehicle
EGF
IGF-1
FGF
HRG-β
(+)Dox
(-)Dox

FIGURE 1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0.1
0
0.0 ng/ml
0.1 ng/ml
1.0 ng/ml
10 ng/ml
0
2
4
6
DAYS OF TREATMENT
Vector #1 - Dox
RELATIVE CELL GROWTH
0.40
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0.20
0.10
0.00
0.0 ng/ml
0.1 ng/ml
1.0 ng/ml
10 ng/ml
0
2
4
6
DAYS OF TREATMENT
TAM67 #62 + Dox
RELATIVE CELL GROWTH
0.40
0.30
0.20
0.10
0.00
0.0 ng/ml
0.1 ng/ml
1.0 ng/ml
10 ng/ml
0
2
4
6
DAYS OF TREATMENT
TAM67 #62 - Dox
RELATIVE CELL GROWTH
0.4
0.3
0.2
0.1
0
0.0 ng/ml
0.1 ng/ml
1.0 ng/ml
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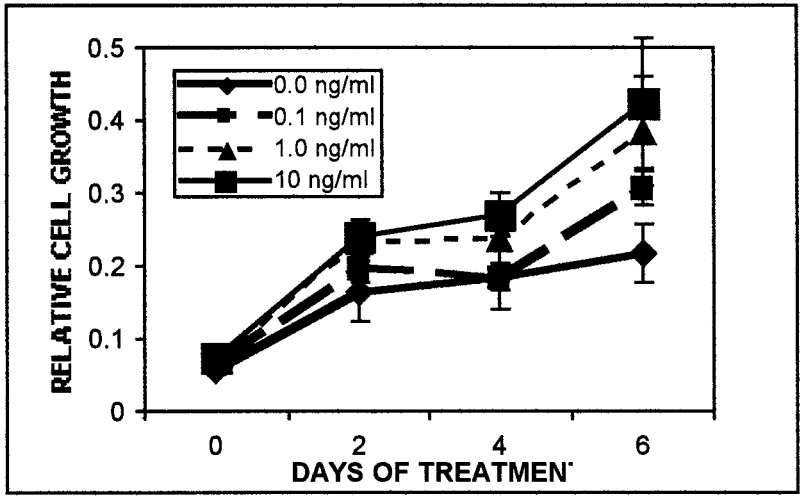
0
2
4
6
DAYS OF TREATMENT

FIGURE 2

FIGURE 3

Vector #1+ Dox



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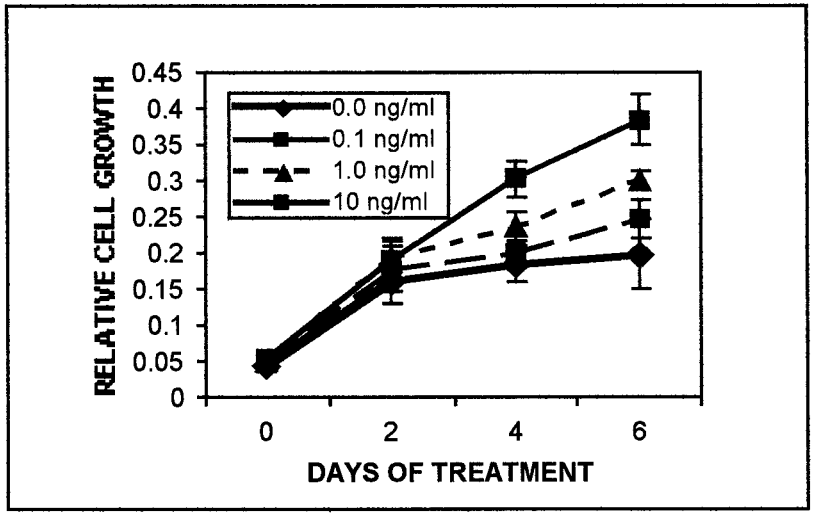

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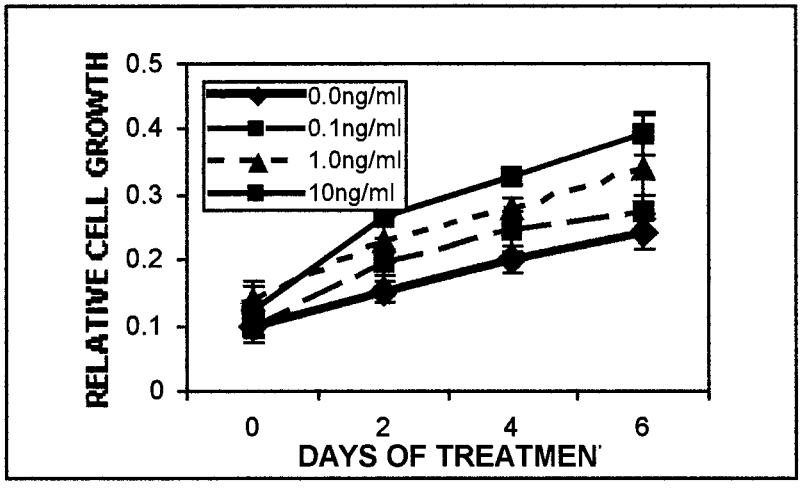

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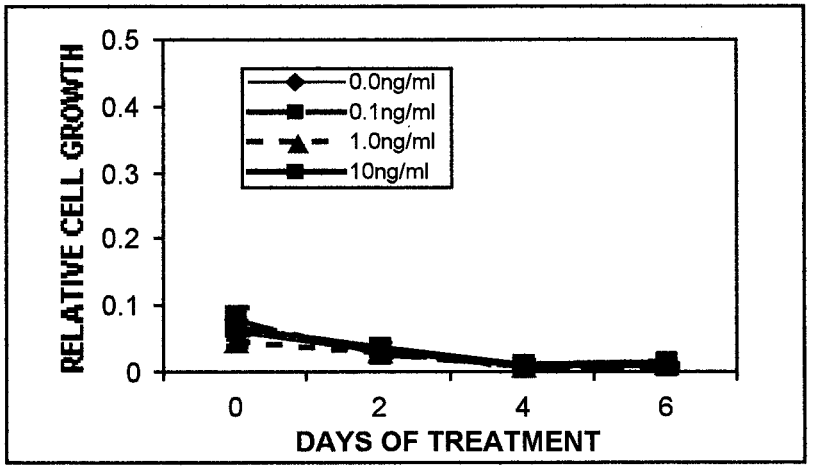

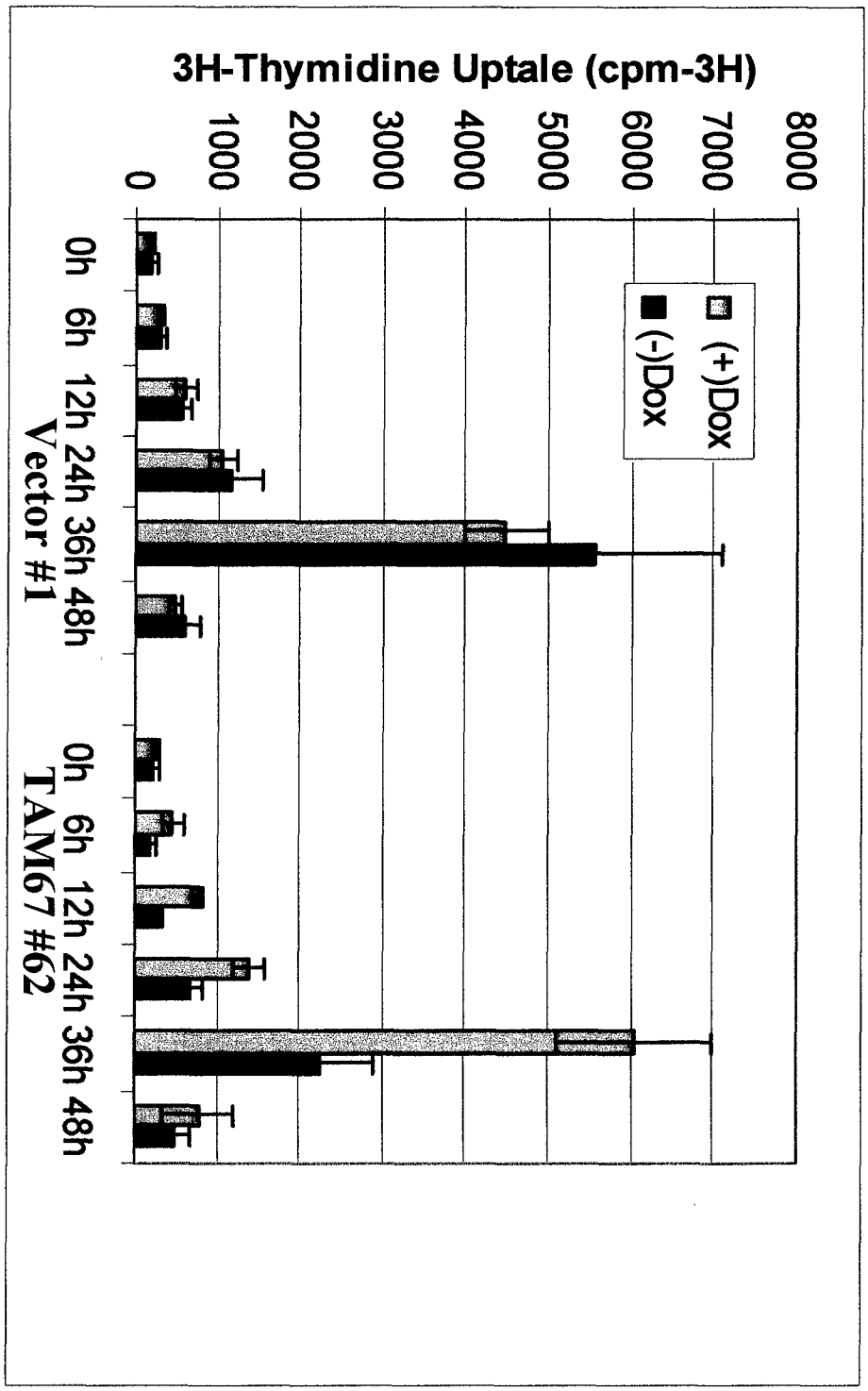
3H-Thymidine Uptale (cpm-3H)
0
1000
2000
3000
4000
5000
6000
7000
8000
(+)Dox
(-)Dox
0h
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12h
24h
36h
48h
TAM67 #62

FIGURE 5

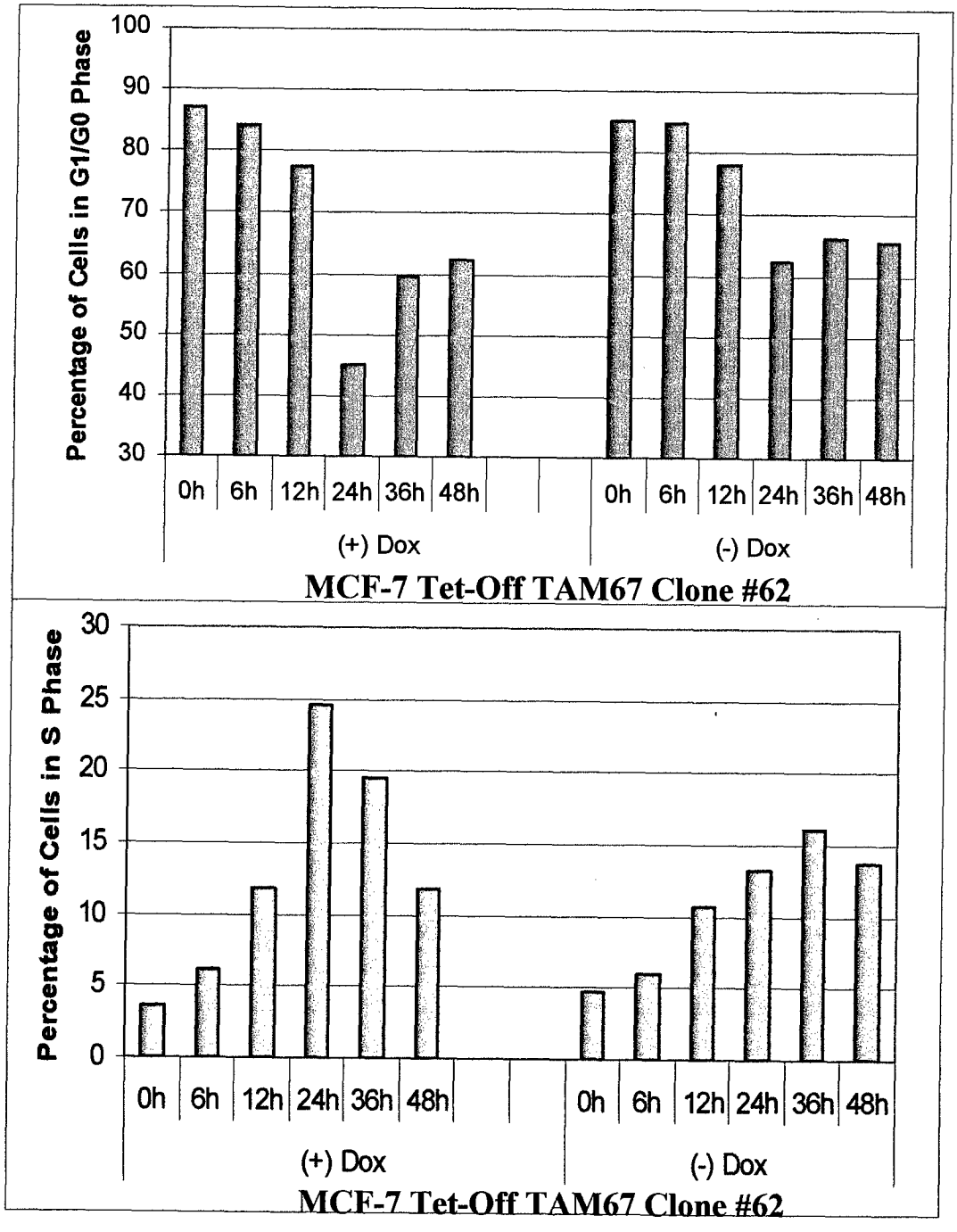
Percentage of Cells in G1/G0 Phase
100
90
80
70
60
50
40
30
0h 6h 12h 24h 36h 48h
0h 6h 12h 24h 36h 48h
(+) Dox
(-) Dox
MCF-7 Tet-Off TAM67 Clone #62
Percentage of Cells in S Phase
30
25
20
15
10
5
0
0h 6h 12h 24h 36h 48h
0h 6h 12h 24h 36h 48h
(+) Dox
(-) Dox
MCF-7 Tet-Off TAM67 Clone #62

Figure 6

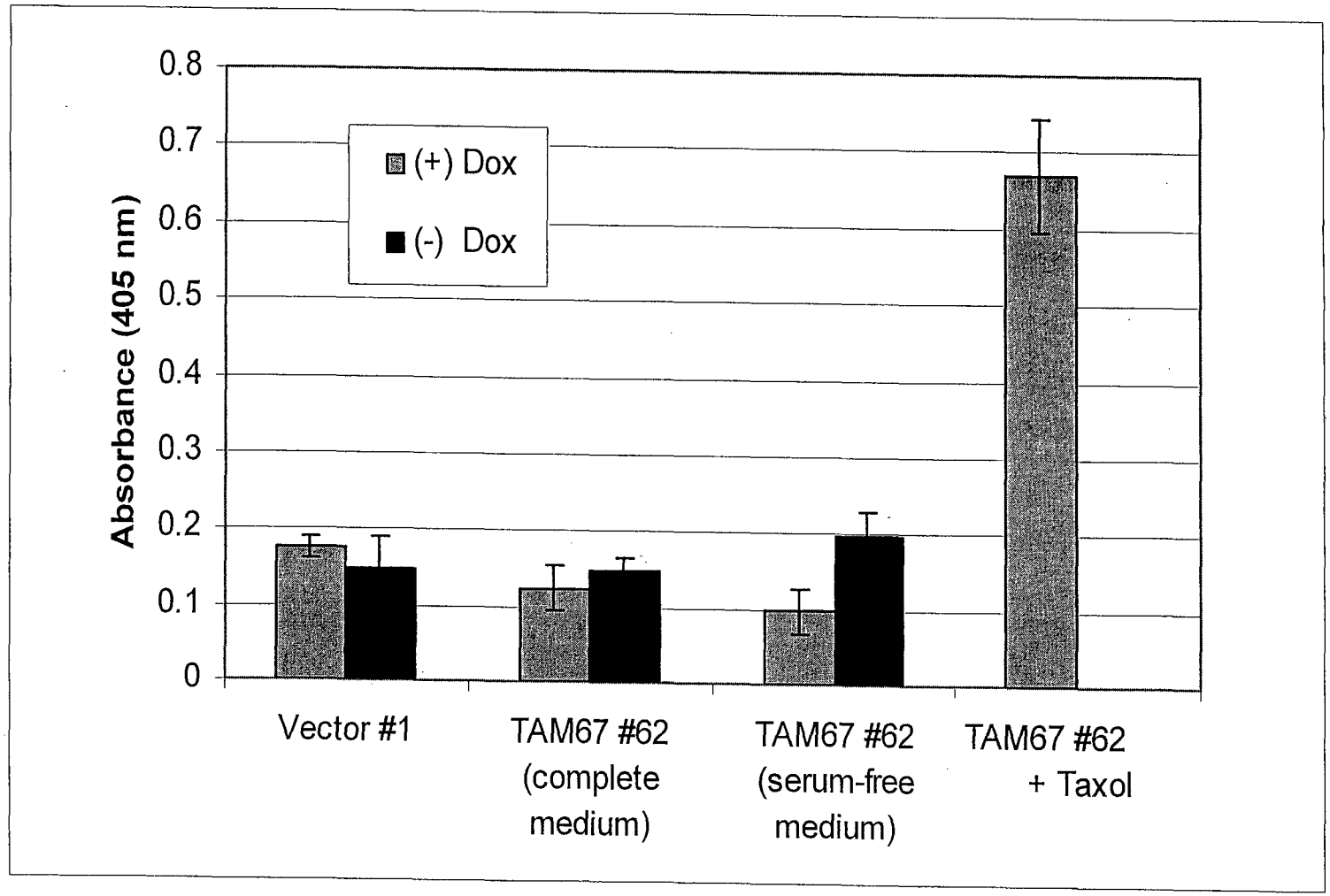

Figure 6: Expression of TAM67 induces apoptosis in serum-free condition

Legends

Fig. 1 Blockade of AP-1 activity induced by different growth factor. MCF-7 Tet-Off TAM67 cells were kept in (+) Dox or (-) medium to block or induce the expression of TAM67. The cells were then transfected with Col-Z-Luc and pRL-TK. The cells were treated with EGF (100 ng/ml), IGF-1 (100 ng/ml), b-FGF (10 ng/ml), Heregulin-β1 (10 ng/ml), or vehicle for 6 hours, respectively. The AP-1 activity was measured using Dual-Luciferase Reporter System (Promega).

Fig. 2 Effect of AP-1 blockade on the heregulin-β induced proliferation of MCF-7 cells. MCF-7 Tet-Off TAM67 clone #62 cells and Vector clone #1 cells were grown in serum-free medium containing 0, 0.1, 1, or 10 ng/ml of heregulin-β1 under un-induced (+Dox) or induced (-Dox) conditions. Cells were harvested every other day and the MTS assay was done according to the protocol provided by manufacturer (Promega). Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Fig. 3 Effect of AP-1 blockade on the FGF induced proliferation of MCF-7 cells. MCF-7 Tet-Off TAM67 clone #62 cells and Vector clone #1 cells were grown in serum-free medium containing 0, 0.1, 1, or 10 ng/ml of b-FGF under un-induced (+Dox) or induced (-Dox) conditions. Cells were harvested every other day and the MTS assay was done according to the protocol provided by manufacturer (Promega). Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Fig. 4 Expression of TAM67 inhibits DNA uptake: ^{3}H-thymidine incorporation assay. MCF-7 Tet-Off TAM67 clone #62 cells and Vector clone #1were grown in (+) Dox or (-) Dox medium to block or induce the expression of TAM67. After synchronization in growth factor-free medium for 24 hours, the cells were labeled with ^{3}H-thumidine (2 μCi/ml) for 1 hour. The cells were lysed, and the ^{3}H-thymidine uptake was measured in a scintillation counter. Each data point was performed in sextuplet, and the results were reported as cpm +/- standard error.

Fig. 5 Expression of TAM67 blocks normal cell cycle: flow cytometry assay. MCF-7 Tet-Off TAM67 cells were grown in (+) Dox or (-) Dox medium to block or induce the expression of TAM67. 24 hours before harvest, the medium is changed to serum-free medium to synchronize the cells. The cells were harvested at time point 0 h, 6 h, 12 h, 24h, 48 h, and were stained with propidium iodide. Stained cells were analyzed with EPICS XL-MCL flow cytometer (Coulter Co.). Histograms were then analyzed for cell cycle compartments.

Fig. 6 Expression of TAM67 induces apoptosis in serum-free condition. MCF-7 Tet-Off TAM67 clone #62 cells and Vector clone #1were grown in (+) Dox or (-) Dox medium to block or induce the expression of TAM67. The experiment was carried out in complete medium and serum free medium. This apoptosis assay measures cytoplasmic DNA fragments and was performed according to Cell Death ELISA Kit (Roche). Each sample was performed in triplets and the results was expressed at mean +/- standard error.

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APPENDIX I

Publications

Award Number DAMD 17-96-1-6225

"Prevention of Breast Cell Transformation by Blockade of the AP-1 Transcription Factor"

Oxidative Stress and AP-1 Activity in Tamoxifen-Resistant Breast Tumors *In Vivo*

Rachel Schiff, Praveen Reddy, Markku Ahotupa, Ester Coronado-Heinsohn, Matt Grim, Susan G. Hilsenbeck, Richard Lawrence, Susan Deneke, Rafael Herrera, Gary C. Chamness, Suzanne A. W. Fuqua, Powel H. Brown, C. Kent Osborne

Background: **Most breast cancers, even those that are initially responsive to tamoxifen, ultimately become resistant. The molecular basis for this resistance, which in some patients is thought to involve stimulation of tumor growth by tamoxifen, is unclear. Tamoxifen induces cellular oxidative stress, and because changes in cell redox state can activate signaling pathways leading to the activation of activating protein-1 (AP-1), we investigated whether tamoxifen-resistant growth *in vivo* is associated with oxidative stress and/or activation of AP-1 in a xenograft model system where resistance is caused by tamoxifen-stimulated growth.** ***Methods:*** **Control estrogen-treated, tamoxifen-sensitive, and tamoxifen-resistant MCF-7 xenograft tumors were assessed for oxidative stress by measuring levels of antioxidant enzyme (e.g., superoxide dismutase [SOD], glutathione *S*-transferase [GST], and hexose monophosphate shunt [HMS]) activity, glutathione, and lipid peroxidation. AP-1 protein levels, phosphorylated c-jun levels, and phosphorylated Jun NH_2-terminal kinase (JNK) levels were examined by western blot analyses, and AP-1 DNA-binding and transcriptional activities were assessed by electrophoretic mobility shift assays and a reporter gene system. All statistical tests are two-sided.** ***Results:*** **Compared with control estrogen-treated tumors, tamoxifen resistant tumors had statistically significantly increased SOD (more than threefold; P = .004) and GST (twofold; P = .004) activity and statistically significantly reduced glutathione levels (greater than twofold; P<.001) and HMS activity (10-fold; P<.001). Lipid peroxides were not significantly different between control and tamoxifen-resistant tumors. We observed no differences in AP-1 protein components or DNA-binding activity. However, AP-1-dependent transcription (P = .04) and phosphorylated c-Jun and JNK levels (P<.001) were statistically significantly increased in the tamoxifen-resistant tumors.** ***Conclusion:*** **Our results suggest that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with oxidative stress and the subsequent antioxidant response and with increased phosphorylated JNK and c-Jun levels and AP-1 activity, which together could contribute to tumor growth. [J Natl Cancer Inst 2000;92: 1926–34]**

Tamoxifen is the most prescribed drug for the prevention and treatment of breast cancer *(1,2)*. However, in breast cancer patients, the disease eventually progresses with the emergence of tamoxifen-resistant tumor cells. Tamoxifen is thought to act primarily by competitive blockade of the estrogen receptor (ER) *(3,4)*. Experimental and clinical evidence suggests that an important form of tamoxifen resistance is the acquired ability of the tumor cells to be stimulated, rather than inhibited, by the drug after prolonged treatment *(5–9)*.

We have developed an *in vivo* experimental model for tamoxifen resistance using ER-positive MCF-7 human breast cancer cells grown in athymic nude mice *(5)*. Tamoxifen treatment suppresses tumor growth for several months, but growth eventually resumes as the tumors become stimulated by tamoxifen *(5)*. The mechanisms underlying the conversion from growth suppression to growth stimulation are still unclear. However, several studies using the MCF-7 *in vivo* model have already discarded a number of potential mechanisms for growth stimulation by tamoxifen, including altered tamoxifen uptake or metabolism *(6,10)* and lost or altered ER *(11)*.

Another possible mechanism for growth stimulation by tamoxifen is an altered intracellular redox status leading to activation of downstream signaling pathways. Cellular redox status is a balance between the rate of pro-oxidant generation, either exogenous or endogenous, and the cellular enzymatic and nonenzymatic antioxidant capacities. A number of studies *(12–16)* have shown that, depending on the cellular microenvironment, tamoxifen can affect the intracellular redox status as either a pro-oxidant or an antioxidant. For example, tamoxifen has the ability to protect lipids, proteins, and DNA against oxidative damage *(13)* and can itself be activated into reactive electrophilic metabolites *(14)*. Moreover, evidence suggests that tamoxifen can induce phase I and phase II metabolizing enzymes *(15)*, which may contribute to its beneficial antioxidant activity but may also be responsible for its own activation. It is also known that changes in the intracellular redox status can lead to the activation of important transcription factors, including activating protein-1 (AP-1) *(17,18)*.

AP-1 is a heterodimeric transcription factor that is composed of various members of the Jun and Fos families *(19)* and binds to DNA at specific AP-1 binding sites. AP-1 activity is determined in part by phosphorylation of these complex components. Importantly, the transcriptional activity of c-Jun is increased by phosphorylation by the Jun NH_2-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs), which are preferentially activated by a variety of environmental and cellular stresses *(20)*, including oxidative stress *(21)*. AP-1 activity can also be coregulated by protein–protein interactions between AP-1 and the ER *(22)*. Furthermore, tamoxifen can function as an agonist in coactivating ER/AP-1 on promoters regulated by AP-1 sites *(22–26)*.

The observation that AP-1 is important in several mitogenic signaling pathways *(27,28)* led us to hypothesize that an increase in cellular AP-1 activity, perhaps resulting from tamoxifen-induced oxidative stress and the changes in intracellular redox status, may contribute to the devel-

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See "Notes" following References."

opment of tamoxifen-resistant tumor growth. In this study, we looked for evidence of oxidative stress and changes in AP-1 activity in the MCF-7 *in vivo* nude mouse model of tamoxifen resistance.

Materials and Methods

Breast Cancer Cells

ER-positive MCF-7 human breast cancer cells (originally obtained from Dr. H. Degani at the Weizmann Institute of Science, Rehovoth, Israel) were used for all experiments, unless otherwise stated. Tissue culture methods have been described previously *(29)*. Exponentially growing cultures were treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (50 ng/mL) in the presence of serum-containing medium for the indicated times. A doxorubicin-resistant subclone of MCF-7, MCF-7 Adria (obtained from Dr. K. Cowan, National Cancer Institute, Bethesda, MD), known to express high levels of c-Jun, was used as an internal standard in the electrophoretic mobility shift assay. ER-negative human MDA-MB-435 breast cancer cells were cultured as described previously *(30)* and were used to obtain ER-negative xenograft tumors.

Athymic Nude Mouse Model of Tamoxifen-Stimulated Growth

Animal care was in accordance with institutional guidelines. Four- to 6-week-old female ovariectomized BALB/c athymic nude mice (Harlan Sprague-Dawley Inc., Madison, WI) were given a subcutaneous injection in the mammary fat pad of 5×10^6 MCF-7 cells or their transfectant derivatives (*see below*) and hormonally treated as described previously *(5,7)*. Estradiol pellets (0.25 mg; Innovative Research, Rockville, MD) were placed subcutaneously in the interscapular region of the mice to stimulate tumor growth. When tumors reached a diameter of 8–12 mm (2–4 weeks), each mouse was randomly allocated to one of the following four groups: 1) control estrogen-treated, 2) removal of the estrogen pellet (i.e., estrogen withdrawal only), 3) estrogen withdrawal and treatment with 500 μg of tamoxifen citrate (AstraZeneca, Macclesfield, U.K.) in peanut oil (subcutaneously injected daily Monday through Friday), or 4) estrogen withdrawal and treatment with 5 mg of ICI 182,780 (AstraZeneca) in castor oil (subcutaneously injected weekly). Tumor growth was assessed and tumor volumes were measured as described previously *(29)*.

Tumors were removed during estrogen treatment (control estrogen-treated tumors) and at various times after the treatment with the antiestrogen drugs tamoxifen and ICI 182,780. Antiestrogen-sensitive tumors are defined as those harvested during the first 3 months of treatment when tumor growth is inhibited by tamoxifen. Thus, these tumors are defined as tamoxifen sensitive (tamoxifen^S) and ICI 182,780 sensitive (ICI^S), respectively. Tumors were usually harvested 2 weeks after the initiation of treatment unless otherwise stated. For the DNA-binding studies, tumors were also harvested at 1, 2, and 3 months after tamoxifen treatment began. After 3–5 months of continuous treatment, growth resumes and tumor progression is evident as an increase in tumor volume. Tumors that first undergo growth inhibition and then resume growth after prolonged antiestrogen treatment are defined as tamoxifen resistant (tamoxifen^R) or ICI 182,780 resistant (ICI^R). We have shown previously that tamoxifen resistance in this model is due to tamoxifen-stimulated tumor growth *(5,7)*.

In the estrogen-withdrawal group of mice, tumors were removed after 2 weeks (estrogen withdrawal^S) or several months later after tumor growth resumed (estrogen withdrawal^R). Each tumor analyzed was from a different mouse; tumor tissues were removed from each mouse and kept at −190 °C for later analyses.

Antioxidant Enzyme Assays

Antioxidant enzyme activities were assessed in the control (estrogen-treated), tamoxifen^S, and tamoxifen^R groups (five tumors per group). Homogenates of frozen tumors (20 [wt/vol]) were prepared in a 0.25 *M* sucrose solution (0 °C) with a Potter–Elvehjem glass–Teflon homogenizer driven by an electric drill at 500 rpm with pulse homogenization six times at 20-second intervals. Homogenates were centrifuged for 10 minutes at 10 000*g* at 4 °C to remove nuclei, mitochondria, and lysosomes, and the supernatants were collected. The activities of superoxide dismutase (SOD) (Cu/Zn form) and catalase were measured in the tumor homogenates, and the activities of glutathione *S*-transferase (GST) and the hexose monophosphate shunt (HMS) were measured in the 10 000*g* supernatants. Enzyme activities were assayed with optimal incubation times and protein concentrations to ensure the linearity of the reaction velocity. SOD activity (μg/mg protein) was measured by luminometric detection of the superoxide anion produced in the xanthine–xanthine oxidase system *(31)*. Catalase activity (μg/mg protein) was determined spectrophotometrically by measuring the rate of disappearance of H_2O_2 *(32)*. GST activity (nmol · min^{-1} · mg^{-1} protein) was measured spectrophotometrically with 1-chloro-2,4-dinitrobenzene as the substrate *(33)*. HMS activity (nmol · min^{-1} · mg^{-1} protein) was assessed spectrophotometrically by the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH) with the use of glucose 6-phosphate as the substrate *(34)*; HMS activity represents the sum of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities.

Lipid Peroxidation and Glutathione Levels

Lipid peroxidation was assessed in tumors from the control estrogen-treated, tamoxifen^S, and tamoxifen^R groups (five tumors per group) by the quantitation of the appearance of conjugated diene double bonds in lipid extracts *(35)*. Briefly, lipids were extracted from 10 000*g* supernatants with chloroform–methanol, dried under a nitrogen atmosphere, redissolved in cyclohexane, and analyzed spectrophotometrically at 233 nm to quantify diene conjugation as detected by peak absorption. Lipid peroxidation is expressed as ΔAbs/mg protein, where ΔAbs is the difference in absorbance between the sample and the cyclohexane solvent.

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels (μmol/g wet wt) were determined from the control estrogen-, tamoxifen-, estrogen-withdrawal-, and ICI 182,780-treated tumors (four tumors per group). The glutathione content of tumor cytosol supernatants was estimated by high-performance liquid chromatography *(36)*. Protein content was measured by the Bradford method (Bio-Rad Laboratories, Richmond, CA).

Protein Extraction and Western Blot Analysis of Phosphorylated c-Jun and JNKs/SAPKs Forms

Phosphorylated forms of c-Jun and JNKs/SAPKs were analyzed with the use of PhosphoPlus antibody kits (Cell Signaling, Inc., Beverly, MA) according to the manufacturer's directions. Briefly, pellets of *in vitro* cultured cells or ground, frozen tumor powders from the control estrogen-treated, tamoxifen^S, and tamoxifen^R groups (at least eight tumors per group) were manually homogenized in lysis buffer (Cell Signaling, Inc.). After microcentrifugation at 14 000*g* for 30 minutes at 4 °C, supernatants were collected, and the protein concentration was determined. Aliquots (25 μg) of protein from each sample were separated under denaturing conditions by electrophoresis with 10% polyacrylamide gel containing sodium dodecyl sulfate and transferred by electroblotting onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The blots were stained with StainAll Dye (Alpha Diagnostic International, Inc., San Antonio, TX) to confirm uniform protein transfer. Separate membranes were then reacted with either c-Jun- or JNK/SAPK-specific PhosphoPlus antibodies that specifically recognize the phosphorylated forms. The membranes were stripped and reblotted for total c-Jun and JNK/SAPK with antibodies that recognize the respective proteins independently of their phosphorylation status. Blots were developed by chemiluminescence (Cell Signaling, Inc.). Each sample was analyzed twice on separate immunoblots. Bands were quantified by densitometric scanning of developed films with the use of the Image 1.61/ppc software program of the National Institutes of Health, Bethesda, MD.

Electrophoretic Mobility Shift Assay

Nuclear extracts from cells or individual tumors (five tumors per group) were prepared as described previously *(28)* with minor changes. Briefly, cells or ground-up, frozen tumor powders were disrupted in lysis buffer (i.e., 10 m*M* HEPES, 1 m*M* EDTA, 60 m*M* KCl, 0.5 m*M* dithiothreitol [DTT], 0.5% Nonidet P-40 [NP-40], and protease inhibitors [1 m*M* phenylmethyl sulfonyl fluoride, 0.4 μ*M* aprotinin, and 10 μ*M* leupeptin]), and nuclei were isolated by microcentrifugation at 2500*g* at 4 °C for 10 minutes. The isolated nuclei were lysed by three cycles of freezing/thawing in nuclear suspension buffer (250 m*M* Tris [pH 7.8], 400 m*M* KCl, 0.5 m*M* DTT, 20% glycerol, and protease inhibitors) and microcentrifuged at 16 000*g* at 4 °C for 10 minutes, and the supernatants (nuclear protein extracts) were collected. Protein concentrations were determined with the use of the Bradford method. Electrophoretic mobility shift assays were performed with 10 μg of nuclear protein extract in a 20-μL reaction mixture containing 20 m*M* HEPES (pH 7.9), 40 m*M* KCl, 1

mM EGTA [i.e., ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid], 1 mM phenylmethyl sulfonyl fluoride, 0.5 mM DTT, 1% glycerol, 2 μg of poly (dI-dC), and 100 pg of [γ-^{32}P]adenosine triphosphate end-labeled, double-stranded oligonucleotide probe containing an AP-1 binding site (5′ CTAGTGATGAGTCAGCCGGATC 3′; Stratagene, La Jolla, CA; the AP-1 binding site is *underlined*). Reaction mixtures were incubated for 30 minutes at room temperature. For oligonucleotide competition experiments, the reaction mixtures were preincubated with a 100-fold excess of unlabeled, cold oligonucleotide probes containing an AP-1 or Sp-1 binding site (Stratagene) for 20 minutes before the addition of the radioactive probes. The reaction mixtures were separated on 5% nondenaturing polyacrylamide gels at 4 °C. After electrophoresis and drying, gels were autoradiographed, and shifted bands were quantified on a PhosphoImager (Molecular Dynamics, Sunnyvale, CA). The AP-1 DNA-binding activity of individual tumors was normalized between gels with the use of an internal standard extract of MCF-7 Adria cells. Electrophoretic mobility shift assays of the same extracts were repeated a minimum of two times.

AP-1/CAT Reporter Constructs, Stable Transfection, and Chloramphenicol Acetyltransferase Assay

To study AP-1-dependent gene transcription, we used a chloramphenicol acetyltransferase (CAT) reporter system as described previously *(37,38)*. The reporter construct, Col-TREx5/TKCAT (TREx5), contains five copies of a consensus AP-1 binding site (5′ ATGAGTCAG 3′) upstream of the herpes simplex virus-thymidine kinase (HSV-tk) minimal promoter. The same site is also a synthetic consensus TPA responsive element (TRE). We also used a control construct, TREΔ-72/TKCAT (TREΔ-72), upstream to position −109 of the HSV-tk promoter, that contains a point mutation in the AP-1 site (5′ TGGAGTCAG 3′) that eliminates both basal and inducible AP-1 activities *(37,38)*.

To generate stable transfection clones, we plated MCF-7 cells at a density of 8×10^5/100 mm^2. After 24 hours, the cells were cotransfected with 10 μg of the TREx5 or TREΔ-72 AP-1/CAT reporter constructs and with 1 μg of the pSV2neo selection plasmid (Clontech Laboratories, Inc., Palo Alto, CA) containing the neomycin resistance gene with the use of the LipofectAMINE reagent (Life Technologies, Inc. [GIBCO BRL], Rockville, MD), according to the manufacturers directions. G418-resistant (600 μg/mL) individual clones were screened for TPA-inducible AP-1 transcriptional activity (50 ng/mL TPA for 8 hours), and CAT assays were performed as described below.

TPA-inducible TREx5 clones and noninducible TREΔ-72 clones were grown in nude mice and treated with tamoxifen as described above. Tumors were harvested from the control estrogen-treated, tamoxifen^S, and tamoxifen^R groups (four to eight mice per group). Tumor samples were homogenized, and extracts were made as described previously *(39)*. Protein concentrations were determined, and the same amount of protein from each clone was analyzed for CAT activity by thin-layer chromatography and quantitated on a PhosphoImager (Ambis, San Diego, CA). We calculated the relative CAT activity by dividing the CAT activity of the tamoxifen-treated tumors by the CAT activity of the control estrogen-treated tumors of the clone. All CAT assays were repeated two times with each sample.

Statistical Methods

Differences in the mean values of tumor antioxidant enzyme activities (SOD, HMS, and GST), glutathione levels, DNA-binding activities, and AP-1-dependent CAT activities among the treatment groups were analyzed by Student's *t* test as pairwise comparisons with respect to the control estrogen-treated group or to the tamoxifen^S group, as specified. Bonferroni's correction was used to adjust for multiple comparisons. The mean values of western blot band densities of phosphorylated c-Jun and JNKs/SAPKs were compared by two-way analysis of variance. For purposes of statistical analyses, data were transformed by taking logarithms to equalize variances. All *P* values are two-sided.

RESULTS

Antioxidant Enzyme Activity

We have developed and studied an experimental *in vivo* model that mimics the clinical scenario of acquired resistance of breast cancer to tamoxifen or other endocrine therapies, such as estrogen-withdrawal or ICI 182,780 treatment *(7)*. In the nude mouse model, ER-positive MCF-7 xenograft tumors are established in the presence of estrogen (control estrogen-treated tumors), and the estrogen is withdrawn before the start of any antiestrogen treatment. Tumor growth is initially inhibited, but it eventually resumes after continued treatment. Tumors inhibited by tamoxifen are defined as tamoxifen-sensitive (tamoxifen^S) tumors, and tumors that resumed growth are defined as tamoxifen-resistant (tamoxifen^R) tumors. We have shown previously that tamoxifen stimulates tamoxifen^R tumor growth *(5,7)*. In parallel, tumors inhibited by estrogen withdrawal or ICI 182,780 are defined as estrogen-withdrawal^S or ICI^S tumors, respectively, and tumors that resumed growth are defined as estrogen-withdrawal^R or ICI^R tumors, respectively.

To investigate the relationship between tamoxifen resistance and altered cellular redox status, we first measured the activity levels of different antioxidant enzymes in control estrogen-treated, tamoxifen^S, and tamoxifen^R tumors grown in nude mice (Table 1). SOD activity was notably increased by tamoxifen, with a greater than fourfold increase in tamoxifen^S tumors (P<.001) and a greater than threefold increase in tamoxifen^R tumors (P = .004). Catalase activity was not statistically significantly different among the tumor groups. GST activity was statistically significantly increased in the tamoxifen^R tumors (twofold; P = .004) relative to the control estrogen-treated or the tamoxifen^S tumors. Tamoxifen^R tumors also had increased protein levels of GST-Pi, a member of the GST enzyme complex, as measured by western blot analysis (data not shown).

The most striking effect of tamoxifen was a profound inhibition of the production of NADPH by the HMS (Table 1). In tamoxifen^S tumors, the HMS activity was statistically significantly reduced to less than half the activity detected in the control estrogen-treated tumors (P<.001). In tamoxifen^R tumors, the HMS activity was further statistically significantly reduced by another fourfold to 10-fold in total (P<.001). Thus, tamoxifen treatment and the development of tamoxifen^R by MCF-7 breast tumors *in vivo* are associated with changes in antioxidant activities, suggesting that the tumors are experiencing oxidative stress.

Oxidative Stress

Lipid peroxidation is a process generated by the effect of reactive oxygen species and occurs when the antioxidant defense mechanisms are being overwhelmed *(40)*. Glutathione, via its redox cycling, is a potent antioxidant that provides cells with a substantial degree of protection against oxidative stress *(41)*. Because decreased HMS activity and NADPH levels would be expected to greatly reduce glutathione levels, we next measured lipid peroxidation and glutathione levels (Table 1). Lipid peroxidation was statistically significantly higher in the tamoxifen^S tumors (P = .016) but then returned to baseline levels after resistance emerged (Table 1). In contrast, levels of both GSH and GSSG were markedly reduced in the tamoxifen^R tumors as compared with the control estrogen-treated tumors (greater than twofold; P<.001 and P = .02, respectively). GSH levels were decreased only slightly in tamoxifen^S tumors compared with those in control estrogen-treated tumors, and GSSG levels were reduced by 1.7-fold in tamoxifen^S tumors compared with those in control estrogen-treated tumors. Importantly, there were marked and statistically significant differences in the GSH levels between the tamoxifen^S and tamoxifen^R tumors (P<.001). These results suggest that the

Table 1. Antioxidant enzyme activity, lipid peroxidation, and glutathione levels in hormonally treated MCF-7 tumors*

Tumor group	Mean (95% confidence interval)						
	SOD,† μg/mg protein	Catalase,† μg/mg protein	GST,† nmol/min per mg protein	HMS,† nmol/min per mg protein	LPO,† ΔAbs/mg protein	GSH,‡ μmol/g protein	GSSG,‡ μmol/g protein
E_2	1.25 (0.29–2.21)	1.74 (1.33–2.15)	0.93 (0.60–1.26)	12.10 (10.34–13.86)	717 (488–946)	2.725 (2.692–2.758)	0.434 (0.279–0.589)
Tam^S	5.01 (3.76–6.26)	2.38 (1.69–3.07)	0.97 (0.77–1.17)	5.42 (4.66–6.18)	2217 (1243–3191)	2.391 (2.322–2.460)	0.248 (0.179–0.317)
Tam^R	4.34 (3.54–5.15)	2.61 (1.77–3.45)	1.83 (1.50–2.16)	1.30 (1.03–1.57)	874 (504–1244)	1.297 (1.281–1.313)	0.156 (0.103–0.209)
$-E_2^R$	ND	ND	ND	ND	ND	2.162 (2.101–2.223)	0.563 (0.357–0.769)
ICI^S	ND	ND	ND	ND	ND	2.002 (1.975–2.029)	0.301 (0.242–0.360)
ICI^R	ND	ND	ND	ND	ND	2.139 (2.053–2.225)	0.250 (0.179–0.321)

*SOD = superoxide dismutase (Cu/Zn form); GST = glutathione *S*-transferase; HMS = hexose monophosphate shunt; LPO = lipid peroxidation; GSH = reduced glutathione; GSSG = oxidized glutathione; ND = not done; E_2 = control estrogen tumors; Tam^S = tamoxifen-sensitive tumors; Tam^R = tamoxifen-resistant tumors; $-E_2^R$ = estrogen-withdrawal resistant tumors; ICI^S = ICI 182,780-sensitive tumors; ICI^R = ICI 182,780-resistant tumors.

†Tumors from five mice per treatment group. ΔAbs = difference in absorbance between the sample and the cyclohexane solvent.

‡Tumors from four mice per treatment group.

development of tamoxifen resistance in MCF-7 breast tumors *in vivo* is associated with increased susceptibility to oxidative stress and depletion of glutathione levels as the tumors attempt to respond to the oxidative stress.

To confirm that the marked decrease in total glutathione levels in resistant tumors was specific for tamoxifen, we measured glutathione levels in tumors from mice treated either with estrogen withdrawal or with the estrogen antagonist ICI 182,780. Although, compared with the levels in control estrogen-treated tumors, glutathione levels decreased slightly in tumors that had acquired resistance to estrogen withdrawal and in ICI^S or ICI^R tumors, glutathione levels in the $tamoxifen^R$ tumors were still substantially lower than those in all other tumors.

To learn whether tamoxifen-induced oxidative stress is mediated though the ER, we measured total glutathione levels in xenograft tumors of ER-negative MDA-MB-435 human breast cancer cells. Tamoxifen does not inhibit *in vivo* growth of this cell line, and long-term experiments are not possible because the tumors become too large. Tamoxifen treatment for 29 days resulted in no reduction in glutathione levels in these tumors (data not shown), suggesting that the effect of tamoxifen might be mediated through the ER.

Tamoxifen Resistance and AP-1 DNA-Binding Activity

Because oxidative stress has been shown to activate the AP-1 transcription factor, which increases cell proliferation *(27,28)*, we explored the effect of tamoxifen on AP-1 composition and activity in MCF-7 tumors *in vivo*. AP-1 is a heterodimeric transcription factor complex that is composed of proteins from the Jun and Fos families. Comparison of control estrogen-treated, $tamoxifen^S$, and $tamoxifen^R$ tumors revealed that there were no apparent changes in messenger RNA or protein levels of the Jun and Fos family members c-Jun, JunD, JunB, Fra-1, and c-Fos (data not shown).

Using electrophoretic mobility shift assays, we next compared AP-1 DNA-binding activity in nuclear extracts from control estrogen-treated, $tamoxifen^S$, and $tamoxifen^R$ tumors (Fig. 1, A). $Tamoxifen^S$ nuclear extracts were made from established tumors 2 weeks, 1 month, 2 months, and 3 months after tamoxifen treatment began. Although there was a modest reduction in AP-1 DNA-binding activity during the first 2 months of tamoxifen treatment, there were no statistically significant differences in AP-1 DNA-binding activity between control estrogen-treated tumors and tamoxifen-treated tumors at any time (Fig. 1, A). DNA-binding assays performed with AP-1 oligonucleotides containing one or five copies of the AP-1 site produced comparable results (data not shown). Using specific antibodies to various AP-1 family members (c-Jun, JunD, Fra-1, and c-Fos), we determined the composition of the AP-1 DNA-binding complexes. We found no appreciable differences in the composition of the DNA-binding complexes among any of the control or tamoxifen-treated groups (data not shown).

Although tamoxifen treatment did not appear to change AP-1 DNA-binding activity, we detected a fourfold decrease in AP-1 DNA-binding activity in estrogen-$withdrawal^S$ tumors ($P<.001$) and in estrogen-$withdrawal^R$ tumors ($P<.001$) (Fig. 1, B). We observed a 3.5-fold decrease in AP-1 DNA-binding activity in ICI^R tumors ($P<.001$). One aberrant tumor in the ICI^S treatment group did not show a decrease in AP-1 DNA-binding activity. Thus, although tamoxifen, estrogen withdrawal, and ICI 182,780 all inhibited tumor growth *in vivo*, only tamoxifen maintained AP-1 DNA-binding activity at initial levels.

Association of Increased AP-1 Transcriptional Transactivating Activity With Development of $Tamoxifen^R$ Growth

AP-1 DNA-binding activity does not necessarily reflect the transcriptional activity of this transcription factor complex *(42)*. To determine whether AP-1 DNA-binding activity is a direct reflection of its ability to promote transcription in this system, we developed stable transfectants of MCF-7 cells expressing CAT reporter gene constructs containing either five copies of a synthetic AP-1 DNA-binding site (TREx5) or a control mutated AP-1 DNA-binding site lacking basal and inducible AP-1 activities (TREΔ-72) up-

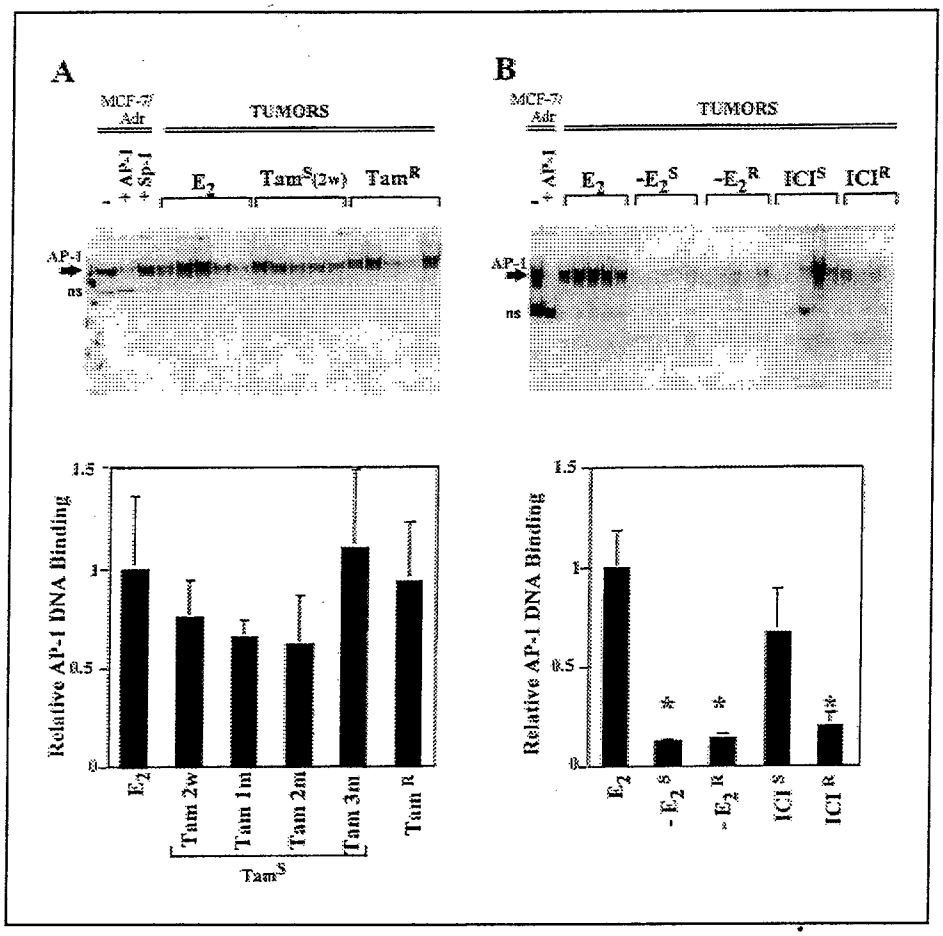

Fig. 1. Activating protein-1 (AP-1) DNA-binding activity of control estrogen-, tamoxifen-, estrogen-withdrawal-, and ICI 182,780-treated MCF-7 breast cancer xenograft tumors. Nuclear extracts from tumor groups (five mice per group) were analyzed by electrophoretic mobility shift assay with the use of an AP-1 oligonucleotide probe. **A)** Tumors from control estrogen-treated (E_2), tamoxifen-sensitive (the groups included 2 weeks [2w], 1 month [1m], 2 months [2m], and 3 months [3m] tamoxifen treatment during the growth-inhibited [Tam^S] phase), and tamoxifen-resistant (during the growth-stimulated [Tam^R] phase) xenograft mice were harvested and analyzed for AP-1 DNA-binding activity. **B)** Tumors from control estrogen-treated (E_2), estrogen-withdrawal-treated ($-E_2$), or estrogen-withdrawal-treated/ICI 182,780-treated xenograft mice at the sensitive, growth-inhibited phase (2 weeks of treatment, $-E_2^S$ or ICI^S) and at the resistant, growth-stimulated phase ($-E_2^R$ or ICI^R) were harvested and analyzed for AP-1 DNA-binding activity. **Top panels:** representative electrophoretic mobility shift assay gels of the tumor nuclear extracts. For Tam^S, only 2 weeks of treatment is shown. An internal standard extract of MCF-7 Adria cells (MCF-7/Adr) was included in each panel for normalization of the AP-1 binding reactions between gels. An excess of unlabeled AP-1 oligonucleotide (+ AP-1) was used to competitively inhibit specific AP-1 binding, and a nonspecific Sp-1 oligonucleotide (+ Sp-1) was added as a further negative control for this specificity. **Arrows** point to the specific AP-1 complexes, and the nonspecific band is designated "ns." **Bottom panels:** relative AP-1 DNA-binding activity in the treated tumors. Specific AP-1 complexes were quantitated on a PhosphoImager, and the DNA-binding activity of individual tumors was normalized between gels. AP-1 DNA-binding activity in the tumor groups was calculated relative to the control estrogen-treated group, and calculated means (±95% confidence intervals) were analyzed statistically by Student's *t* test as pairwise comparisons versus the control estrogen-treated group. * = $P<.001$. Electrophoretic mobility shift assays with the same samples were repeated a minimum of two times.

stream to a minimal promoter *(37)*. TPA induced CAT activity *in vitro* in the two TREx5 clones tested but not in the TREΔ-72 clone (Fig. 2, A). The stable transfectants were then grown as tumors in the nude mice and treated with tamoxifen. In tumors grown from the control TREΔ-72 clone, the low basal CAT activity gradually declined during tamoxifen treatment (Fig. 2, B and C). In contrast, a statistically significant increase in CAT activity was detected in tamoxifen^R tumors in both TREx5 clones (twofold to threefold; $P = .04$ and $P = .006$ for TREx5 clones 1 and 2, respectively). The finding that AP-1 transcriptional activity increased at the time of tamoxifen^R growth was reproducible in two different clones and in two independent *in vivo* experiments.

Hyperphosphorylation of c-Jun in Tamoxifen^R Tumors

AP-1 transcriptional activity is increased by phosphorylation at two specific serine residues in the c-Jun component of AP-1 *(42)*. These residues, Ser 63 and Ser 73, are phosphorylated by the JNKs *(43,44)*. Furthermore, JNK activity can be increased by various stresses *(20)*, including oxidative stress *(21)* and/or glutathione depletion *(45)*, conditions observed in our model with tamoxifen treatment, especially during tamoxifen^R growth.

Because we observed an induction of oxidative stress in the tumors during the emergence of tamoxifen resistance, we determined the phosphorylation status of c-Jun from control estrogen-treated, tamoxifen^S, and tamoxifen^R tumors (Fig. 3, A). No statistically significant changes were detected between the control estrogen-treated tumors and tamoxifen^S tumors. However, the tamoxifen^R tumors (of both untransfected MCF-7 tumors and stable TREx5 transfectants) had statistically significantly higher levels of phosphorylated c-Jun (greater than twofold; $P<.001$), even after correcting for minor changes in total c-Jun levels.

Because the JNKs are the major kinases known to phosphorylate c-Jun at Ser 63 and Ser 73, we next measured phosphorylated (i.e., active) JNK family members in the control estrogen-treated, tamoxifen^S, and tamoxifen^R tumors (Fig. 3, B). We saw that the tamoxifen^R tumors contained statistically significantly higher levels of both the 46-kd and 54-kd phospho-JNK forms compared with the tamoxifen^S tumors (>1.8-fold and 1.5-fold; $P<.001$ and $P = .008$, respectively, for 46-kd and 54-kd phospho-JNK forms, after correcting for minor changes in the level of total JNK). Thus, both increased phospho-c-Jun levels and increased JNK activity accompanied the increase in AP-1-dependent transcription in the tamoxifen^R tumors.

Discussion

The emergence of tamoxifen resistance is a major problem in the treatment of breast cancer, and understanding the mechanisms by which resistance arises could have major clinical implications for preventing or circumventing it. Our results show that the development of acquired tamoxifen resistance of xenograft MCF-7 tumors *in vivo* is associated with both increased susceptibility to oxidative

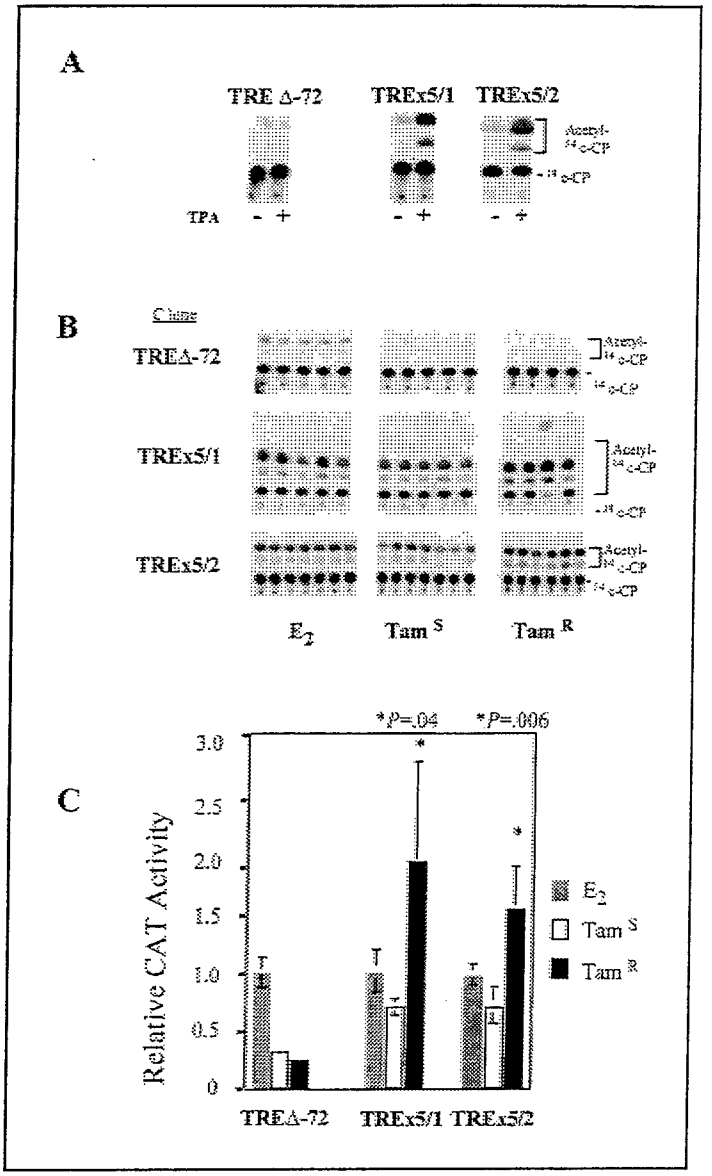

Fig. 2. Activating protein-1 (AP-1) transcriptional activity in tamoxifen-treated tumors. MCF-7 breast cancer cells were stably transfected with chloramphenicol acetyltransferase (CAT) constructs containing either five copies of a consensus AP-1 site (TREx5 clones) or a control mutated AP-1 site (TREΔ-72 construct). **A)** 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) induction of TREΔ-72 and TREx5 clones *in vitro*. Cells of two TREx5 clones (clone TREx5/1 and clone TREx5/2) and one TREΔ-72 clone were left untreated (–) or were treated with TPA (50 ng/mL) for 8 hours, and extracts were analyzed by the CAT assay, as described in the "Materials and Methods" section. In **B** and **C**, AP-1-dependent CAT activity is indicated in tamoxifen-treated tumors. The transfectant clones were injected into nude mice and treated with estrogen and tamoxifen. Tumors (four to eight mice per group) were harvested for CAT analysis after establishment in the presence of estrogen (E_2), 2 weeks after tamoxifen treatment began (during the tamoxifen-inhibited growth phase, Tam^S), and at the appearance of tamoxifen-resistant growth (Tam^R). All tumors were from individual mice. CAT assays were performed with the use of the same amount of protein extract for individual tumors of each clone. **B)** CAT assay of clones TREΔ-72 and TREx5. The TREΔ-72 clone has low basal CAT activity, so its autoradiographs were exposed longer than those of the TREx5 clones. All of the tumors analyzed from the stable transfectants are shown. CAT assays of the same samples were repeated twice. **C)** Quantitation of relative CAT activity of the TRE/CAT clones shown in **B**. The CAT assays were quantitated on a PhosphoImager, and the relative CAT activity in the tumor groups was calculated relative to that of the control estrogen-treated group of each clone. $Tamoxifen^R$ calculated means (±95% confidence intervals) were analyzed statistically by Student's *t* test compared with the $tamoxifen^S$ group. CP = chloramphenicol.

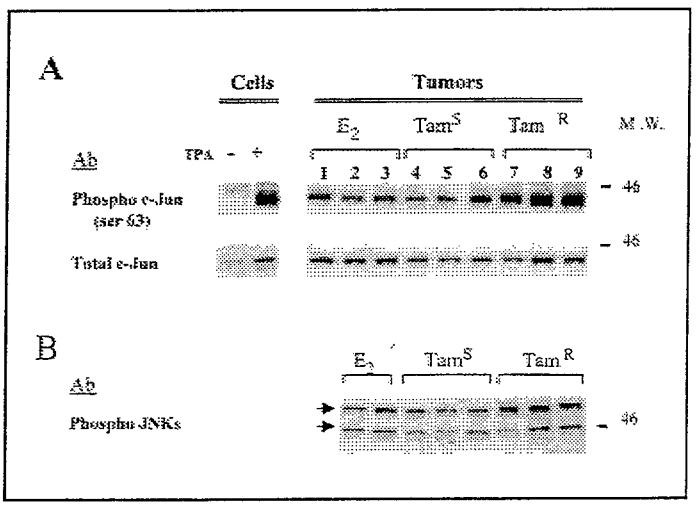

Fig. 3. Phosphorylation of c-Jun and Jun NH_2-terminal kinase (JNK) in the tamoxifen-treated tumors. Protein extracts (25 μg) of control estrogen-treated (E_2), tamoxifen-sensitive (Tam^S), and tamoxifen-resistant (Tam^R) MCF-7 breast cancer xenograft tumors were analyzed by western blot analysis with antibodies that recognize the phosphorylated forms of the proteins. **A)** Blots probed with anti-phospho c-Jun antiserum specific for Ser 63 (**top panel**) and anti-total c-Jun antiserum, which recognizes c-Jun independently of its phosphorylation status (**bottom panel**). Controls were MCF-7 cells either untreated (–) or treated *in vitro* (+) with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) at a dose of 50 ng/mL for 1 hour. **B)** Blots probed with anti-phospho JNK (54 kd and 46 kd) antiserum recognizes Thr 183 and Tyr 185 phosphorylation. **Arrows** point to the 54-kd and 46-kd JNK family members. A representative gel is shown of two or three tumors per group. Mean values of western blot band densities of phosphorylated c-Jun and JNKs/stress-activated protein kinases (SAPKs) were compared by two-way analysis of variance. Ab = antibody; M.W. = molecular weight.

stress and increased AP-1 activity. Tamoxifen affects the intracellular redox status in breast tumors, increases lipid peroxidation, and induces the activity of a number of antioxidant enzymes. Prolonged tamoxifen treatment resulted in tumors that were tamoxifen resistant and growth stimulated and had a reduced antioxidant cellular capacity, as evidenced by a striking decrease in HMS activity and a marked depletion of glutathione. These oxidative changes after prolonged

treatment appear to be specific to tamoxifen because this marked reduction in glutathione levels and, consequently, increased susceptibility to oxidative stress were not found in tumors from mice treated with estrogen, with estrogen withdrawal, or with the pure antiestrogen ICI 182,780.

A remaining open question is to what extent all of the oxidative changes are ER dependent. It is known that tamoxifen's antiangiogenic effects and its potential to induce oxidative stress are, at least in part, ER independent *(46–48)*, although the role of the two ER subtypes is still unclear *(49)*. A recent study *(50)* demonstrating that the oncostatic action of melatonin on breast cancer cells is mediated by increased glutathione levels and is restricted to ER-positive cells suggests a link between cellular redox state and ER. In addition, the lack of oxidative stress in ER-negative MDA-MB-435 tumors from tamoxifen-treated mice and preliminary *in vitro* data also suggest that tamoxifen-induced oxidative stress in MCF-7 tumors may be ER mediated.

Because changes in the cellular redox status can lead to the induction of AP-1 and because AP-1 is important in a variety of mitogenic signaling pathways *(27,28)*, we studied this transcription factor complex in our tamoxifen^R *in vivo* model. We found that the AP-1 transcriptional activity was increased as tumors progressed to a tamoxifen^R phenotype. It is possible that the observed increase in the antioxidant enzyme GST, whose expression is regulated by AP-1 *(51)*, reflects a parallel increase in AP-1-dependent transcription. Other reports have shown that prolonged tamoxifen treatment dramatically affects the expression of a number of phase II enzymes, such as GST *(52–54)*, probably through the antioxidant response element contained in the promoter of these genes *(54)* and that these genes may also be regulated by AP-1 *(51)*. In agreement with our finding, Astruc et al. *(25)* also have reported that prolonged tamoxifen treatment markedly increases the cellular response to inducers of AP-1.

Although we found increased AP-1 transcriptional activity in the tamoxifen^R tumors, neither expression of Jun and Fos family members nor AP-1 DNA-binding activity was altered. These findings are similar to those in other reports *(27,28)*. In contrast, Dumont et al. *(55)* reported that the progression of MCF-7 tumor cells (MCF-WES cells) to a tamoxifen-stimulated/tamoxifen-resistant phenotype is associated with increased AP-1 DNA-binding activity. However, MCF-WES tamoxifen^R tumors have a markedly decreased ER content, and although the tumors are still estrogen sensitive, they are globally resistant to all antiestrogens. In contrast, the tamoxifen^R tumors in this study, which have increased AP-1 transcriptional activity, express high levels of ER, remain estrogen dependent *(5)*, and are growth inhibited by pure steroidal antiestrogens *(6,7)*. Thus, although both types of tamoxifen^R tumors share a common regulatory pathway, namely AP-1, they may differ in how the pathway is activated. Whether these differences in AP-1 activation account for the divergence in their cellular phenotype remains to be investigated.

Our data also demonstrate that increases in JNK activity and c-Jun phosphorylation are associated with the tamoxifen^R phenotype. Increased c-Jun phosphorylation by tamoxifen may potentiate c-Jun transcriptional activity *(42,56)* and could also enhance the agonistic effects of tamoxifen at AP-1 sites, as proposed by Webb et al. *(22)*. JNK can be activated by diverse stress stimuli *(20,42)*, including oxidative stress *(21)*. Oxidative stress can alter the level of intracellular glutathione, which can be a key regulator for the induction of JNKs *(45)*. Because both oxidized and reduced glutathione levels are markedly decreased in our tamoxifen^R tumors but not in our tamoxifen^S tumors, our cumulative data suggest the possibility that chronic tamoxifen administration leads to oxidative stress and a reduction in glutathione levels followed by activation of JNK and increased AP-1 activity. The increased AP-1 activity could then provide the tumor cells with a sufficient growth stimulus to offset any growth-inhibitory effects of the antiestrogen mediated via the ER pathway. Recent data demonstrating a substantial increase in JNK activity in tamoxifen-resistant human breast tumors *(57)* further support this hypothesis.

Although our data show that prolonged tamoxifen treatment of MCF-7 tumors in nude mice results in oxidative stress and increased AP-1 activity, to conclude that these pathways mediate tamoxifen^R growth, it will be necessary to determine whether increasing or inhibiting AP-1 activity or cellular sensitivity to oxidative stress will affect the emergence of the tamoxifen^R phenotype. In this context, it is interesting that overexpression of c-Jun in MCF-7 cells results in a tamoxifen^R phenotype both *in vitro* and *in vivo (58)*. Confirmation of the role of oxidative stress and AP-1 in the development of tamoxifen^R could provide new strategies to delay or even to prevent this important clinical problem.

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NOTES

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AP-1 blockade inhibits the growth of normal and malignant breast cells

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We have previously demonstrated that basal AP-1 transcriptional activity is high in normal human mammary epithelial cells, intermediate in immortal breast cells, and relatively low in breast cancer cells. In this study we investigated whether differences in AP-1 transcriptional activity reflect differences in breast cells' dependence on AP-1 for proliferation. The cJun dominant negative, TAM-67, was used to determine the effect of AP-1 blockade on the growth of normal, immortal and malignant breast cells. We first showed that TAM-67 inhibits AP-1 activity in normal and malignant breast cells. We then determined whether this AP-1 inhibitor affected colony forming efficiency of the immortalized and malignant breast cells. The AP-1 inhibitor reduced colony formation of immortal breast cells by over 50% (by 58% in 184B5 cells and 62% in MCF10A cells), and reduced colony formation in the breast cancer cell line MCF7 by 43%, but did not reduce colony formation in the other breast cancer cell lines (T47D, MDA MB231 and MDA MB 435). We also determined the effect of AP-1 blockade on the growth of normal breast cells using a single cell proliferation assay. Using this assay, the growth of normal breast cells was extremely sensitive to AP-1 blockade, while immortal breast cells were moderately sensitive. We next directly tested the effect of TAM-67 expression on the growth of MCF7 breast cancer cells, using cells stably transfected with TAM-67 under the control of a doxycycline-inducible promoter. Upon induction, TAM-67 was expressed and AP-1 activity was inhibited in these cells. We then measured the growth of these cells in the presence or absence of TAM-67. The results of these studies show that the growth of MCF7 cells was suppressed by the AP-1 inhibitor, TAM-67. These results demonstrate that normal and immortalized breast cells, and some breast cancer cells (such as MCF7), require AP-1 to transduce proliferative signals, while other breast cancer cells (such as T47D, MDA MB 231 and MDA MB 435) do not. These studies suggest that the AP-1 transcription factor is a potential target for future agents for the prevention or treatment of breast cancer.
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Introduction

Breast cancer is one of the most common malignancies in women, and the leading cause of death for women between the ages of 40 and 55 in the United States (Landis *et al.*, 1999). Even with aggressive mammographic screening, adjuvant chemotherapy, and intensive therapy for existing cancer, many of the women who develop breast cancer will die from it. Thus, more effective prevention strategies and treatments are urgently needed.

Unfortunately, little is known about the specific molecular events, which cause the progressive transformation of human breast epithelial cells to malignant breast cancer. Studies of model systems of cancer have revealed that multiple steps are involved in carcinogenesis, including tumor 'initiation' and 'promotion' events (Bishop, 1987). Mutations and deletions within tumor suppressor genes may represent the molecular equivalent of breast cancer 'initiation' events (Malkin *et al.*, 1990; Tripathy and Benz, 1993). However, the molecular mechanism of breast tumor 'promotion' is poorly defined. In model systems (Berenblum and Shubik, 1947), classic tumor promoters induce the proliferation of initiated cells, leading to the progressive outgrowth of fully malignant cells. Such tumor promoters typically activate signal transduction pathways to stimulate cellular proliferation. In human breast cells, the overproduction of growth factors, or aberrant stimulation of growth factor receptors, may be responsible for the promotional phase of breast carcinogenesis (Harris *et al.*, 1993).

Growth factors important for mammary epithelial cells, such as estrogen, EGF, TGF-α, and the IGFs, may all represent tumor promoters of human breast cancer. Thus, drugs that inhibit the ability of estrogen to activate the estrogen receptor (tamoxifen and other antiestrogens) and drugs that block growth factor receptors (such as antibodies specific for the Her2/neu receptor) are now being used to

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treat or prevent breast cancer. However, inhibition of individual signal transduction pathways may be only partially effective, since multiple different signal transduction pathways can stimulate breast cell proliferation. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where multiple mitogenic signals converge. Since transcription factors, the nuclear proteins that control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction pathways, inhibitors of these transcription factors may be more potent inhibitors of breast cell growth.

A key family of transcription factors transducing multiple mitogenic signals is the AP-1 family. These transcription factors are complexes of DNA-binding proteins made up of dimers of Jun and Fos proteins, which bind DNA at specific AP-1 sites and regulate the transcription of AP-1-dependent genes. AP-1 transcription factors are expressed in most cell types and are activated by specific kinases, such as the mitogen-activated and stress-activated kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to light, oxidative stress, tumor promoters such as TPA, or oncogene overexpression or activation (Baselga and Mendelsohn, 1994). Thus, AP-1 is a central component of many signal transduction pathways in many different cell types.

We have previously shown that normal breast cells have high basal levels of AP-1, while breast cancer cells have low basal levels (Chen *et al.*, 1996). We hypothesized that the high basal level of AP-1 present in normal breast cells is required to support the growth of normal cells and that these cells would be more dependent on AP-1 for their growth than breast cancer cells. To investigate the role of AP-1 in controlling breast cell growth we have used normal breast cells, immortal breast cells, and breast cancer cells. We determined the effect of AP-1 blockade on the growth of these different breast cells using the cJun dominant-negative mutant, TAM-67. These studies demonstrate that the growth of normal and immortal human mammary epithelial cells, which have the highest basal AP-1 transcriptional activity, is suppressed by AP-1 blockade. Therefore, normal and immortal breast cells require AP-1 for their growth. Breast cancer cells have lower basal AP-1 transcriptional activity are less sensitive to AP-1 blockade. Of the breast cancer cells tested, MCF7 cells were the most sensitive to the growth suppressive effects of the AP-1 inhibitor. The other breast cancer cells tested, T47D, MDA MB 435, and MDA MB 231, were resistant to the growth suppressive effects of TAM-67. These results demonstrate that the growth of normal breast cells and some breast cancer cells is inhibited by AP-1 blockade, and suggest that AP-1 is a promising target for agents for the prevention or treatment of breast cancer.

Results

Expression of the cJun dominant-negative mutant, TAM-67, in breast cells

To determine whether AP-1 transcription factor complexes are involved in controlling breast cell growth we have investigated the effect of inhibiting AP-1 activity on breast cell proliferation. To block AP-1 activity we used the cJun dominant-negative mutant, TAM-67. TAM-67 is a mutant form of cJun in which the transactivation domain has been deleted leaving the DNA binding and the leucine zipper domains intact (Figure 1a). We and others have used TAM-67 to investigate the role of AP-1 in cell transformation, cell differentiation, and apoptosis in many different cell types (Brown *et al.*, 1993; Chen *et al.*, 1996; Domann *et al.*, 1994; Dong *et al.*, 1994; Ham *et al.*, 1995; Petrak *et al.*, 1994).

Expression of the TAM-67 protein in different breast cells was determined by immunoblot analysis of whole cell extracts of breast cells co-transfected with 0.5 μg of the pCMV-TAM-67 expression vector. TAM-67 protein was detected using antibodies directed against the DNA binding domain of the human cJun protein (Figure 1b). Similar levels of TAM-67 protein were observed in all cell lines, with MDA MB 435 cells showing the lowest level of expression. We then

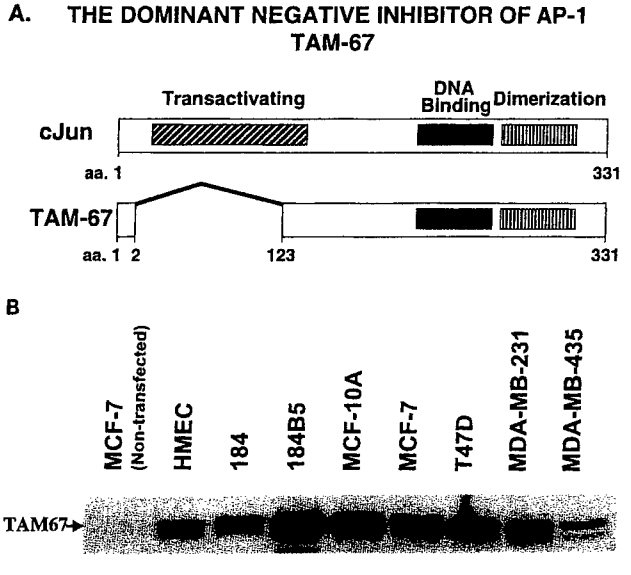

Figure 1 TAM-67, a dominant negative mutant of cJun. (**a**) Schematic representations of the protein functional domains in cJun and the cJun mutant, TAM-67. Three functional domains of cJun are shown: the transactivation domain, the DNA binding domain, and the dimerization domain. TAM-67 has most of the transactivation domain deleted. (**b**) Transient protein expression of TAM-67 in different breast cells. TAM-67 protein expression was determined 36 h after transfection of the different cell lines with 2.5 μg of the TAM-67 expression vector, pCMV-TAM-67. TAM-67 is distinguished from endogenous cJun, because TAM-67 protein is a cJun deletion mutant, which migrates faster than endogenous cJun in the gel. TAM-67 protein was measured in whole cell extracts using Western blot analysis as described in Materials and methods

measured the ability of the TAM-67 protein to inhibit AP-1 activity in these breast cells as shown in Figure 2.

TAM-67 inhibits AP-1 activity in normal and malignant breast cells

To demonstrate that TAM-67 inhibits AP-1 activity in breast cells, we co-transfected the luciferase reporter construct, Col-Z-Luc, with increasing amounts of the TAM-67 expression plasmid, pCMV-TAM-67. The results of these experiments are shown in Figure 2a,b. We observed that the high basal AP-1 activity present in normal and immortal breast epithelial cell is significantly inhibited by TAM-67 expression (Figure 2a). Because basal AP-1 activity is relatively low in breast cancer cells (Chen *et al.*, 1996), we measured the effect of TAM-67 expression on TPA-induced AP-1 transcriptional activity in the breast cancer cells (Figure 2b). Increasing amounts of pCMV-TAM-67 resulted in inhibition of TPA-induced AP-1 activity in all breast cancer cell lines examined. In addition, we have previously observed that TAM-67 inhibits serum-stimulated AP-1 activity in breast cancer cells (Chen *et al.*, 1996). These results demonstrate that TAM-67 inhibits AP-1 transcriptional activating activity in all of the different breast cells.

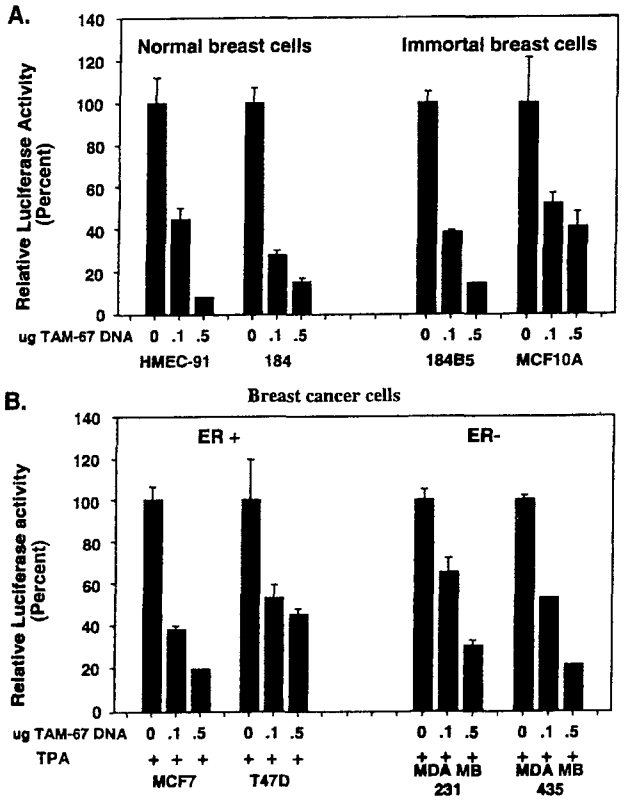

Figure 2 TAM-67 inhibition of Col-Z-Luc activity in all the breast cell lines. (**a**) TAM-67 inhibition of basal Col-Z-Luc promoter activity in normal and immortal human breast cells. Cells were transfected with 1 μg of Col-Z-Luc reporter plasmid plus increasing amounts (0, 0.1 or 0.5 μg) of the expression plasmid pCMV-TAM-67. pCMV (empty vector) was included (0.5, 0.1 or 0 μg) to maintain equal amounts of DNA in each transfection. Transfection procedures were performed as described in Materials and methods. Cells were lysed and luciferase activity was determined 36 h after transfection. (**b**) TAM-67 inhibition of TPA induced Col-Z-Luc promoter activity in breast cancer cells. Cells were transfected with 1 μg of Col-Z-Luc reporter plasmid plus increasing amounts pCMV-TAM-67 as described in (**a**). Induction of AP-1 activity was done 36 h post-transfection by treating the cells with TPA (0.1 nM) for 4–6 h. AP-1 activity was measured as described in Materials and methods

TAM-67 inhibits colony formation of immortal breast cells and some breast cancer cells

We next investigated whether inhibition of AP-1 transcriptional activity affects breast cell proliferation using a colony forming assay. This assay has been extensively used to demonstrate the effects of tumor suppressors and oncogenes on cell growth. As described in Materials and methods, breast cells were co-transfected with pZeoSV and either pCMV vector or pCMV-TAM-67. The pZeoSV plasmid contains a zeocin resistance gene allowing selection of transfected cells. We have measured the effect of TAM-67 on colony formation in immortal and malignant breast cells. Normal mammary epithelial cells could not be analysed because these cells undergo a finite number of doublings and did not form zeocin-resistant colonies. The colony forming results for all cell lines tested are shown in Figure 3. Colony formation of immortal breast cells was reduced when these cells were co-transfected with pCMV-TAM-67 compared to cells co-transfected with pCMV (Figure 3a). The number of colonies was reduced by 62% and by 58% for MCF10A and 184B5, respectively. Colony formation of the MCF7 breast cancer cell line was also reduced (by 43%; see Figure 3b). TAM-67 did not significantly reduce the colony formation in the other breast cancer cell lines. Colony formation of T47D breast cancer cells was not reduced and in fact was significantly increased. These results suggest that TAM-67 is stimulating growth in these cells, either by interacting with a negative growth regulator in T47D cells, or alternatively by activating transcription through interactions with co-activators present in T47D cells.

TAM-67 inhibits normal human mammary epithelial cell growth

To investigate the effect of AP-1 blockade on the growth of normal human mammary epithelial cells we used a second assay, the single cell proliferation assay (SCPA) previously described by Timchenko *et al.* (1996). We used this assay to analyse normal and immortal breast cell growth in the presence of TAM-67. The cells were co-transfected with 5 μg of the expression vector pCMV (empty vector) or pCMV-TAM-67 plus 0.5 μg of pCMV-ß-gal. After allowing recovery from the transfection the cells were plated at low cell densities and cultured to allow single cells to grow into small colonies ranging from 1–20 cells. The

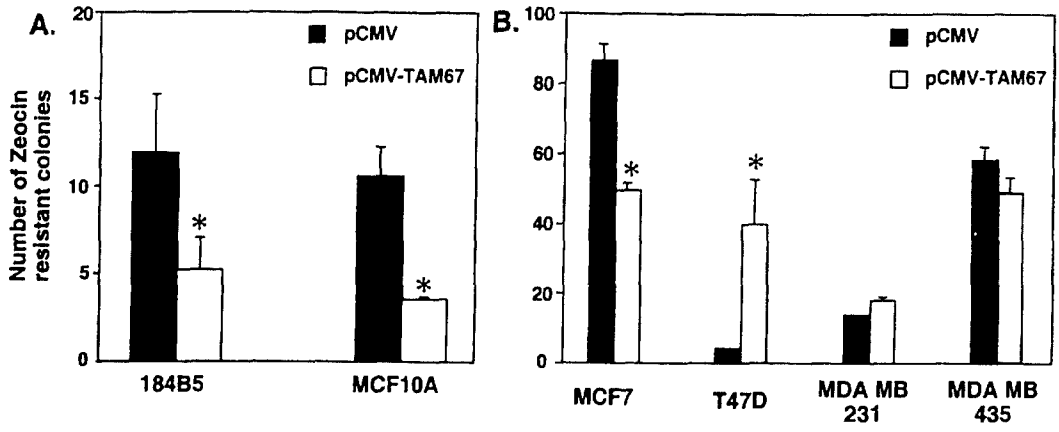

Figure 3 Colony forming efficiency of TAM-67 transfected breast cells. Colony formation of immortalized (a) and malignant (b) breast cells. Immortalized (a) and malignant (b) breast cells were transfected with pSVZco (0.5 μg) and either pCMV (5 μg) or pCMV-TAM67 (5 μg). Transfections were performed as described in Materials and methods. After 2 weeks of selection Zeocin resistant colonies were stained with crystal violet and counted. The data shows the average number of Zeocin resistant colonies from experiments done in triplicate with error bars representing the s.e.m. The names of the different cell lines analysed are given on the x-axis. *P-value < 0.05

cells were then fixed and stained *in situ* for ß-galactosidase activity and transfected cells were identified as blue cells by light microscopy. The number of transfected cells observed per colony were scored and presented as a histogram of the percentage of colonies having 1, 2, 3, or more transfected cells per colony. An example of a blue colony obtained by transfection of breast cells with pCMV-ß-gal is shown in Figure 4a.

Results from these experiments showing the number of cells present in blue colonies in normal and immortal cells transfected with either pCMV or pCMV-TAM-67 is shown in Figure 4b. The upper panels show the histogram obtained when pCMV is transfected into the cells, while the lower panels show the histogram obtained when pCMV-TAM-67 is transfected into the cells. Note that TAM-67 reduces the number of blue cells per colony in both normal cells (HMEC and 184 cells) and immortal breast cells (184B5 and MCF10A). These results demonstrate that the growth of normal and immortal human mammary epithelial cells is inhibited by expression of TAM-67. Also note that the mean number of blue cells per colony is reduced more in normal breast cells (HMEC and 184 cells) than in the immortalized cells (184B5 and MCF10A) (Figure 4b). This result suggests that normal human mammary epithelial cells are more sensitive to the AP-1 inhibitor than are the immortalized breast cells.

Isolation of breast cancer clones expressing TAM-67 under the control of an inducible promoter

To directly investigate whether AP-1 blockade inhibits the growth of breast cancer cells, we created MCF7 cell lines that express TAM-67 under the control of an inducible promoter. The Tet-off system was used for creation of inducible MCF7-TAM-67 and MCF7-vector cell lines. MCF7 tTA cells were transfected with either the empty vector, or with the expression plasmid containing the flag-tagged TAM67 cDNA inserted downstream of a tetracycline-responsive transcriptional promoter as described in Figure 5a. This plasmid was cotransfected with a plasmid carrying the hygromycin resistance gene, allowing for selection of transfected cells. Hygromycin-resistant colonies were then selected under conditions that repress expression of the TAM-67 cDNA. The cells were then screened for inducible TAM-67 protein expression by immunoblotting with anti-cJun antibodies. We used two MCF7 Tet-off TAM-67 clones for further study. Figure 5b shows the inducible expression of these two clones (MCF-7 #62 and MCF7 #67) found to express TAM-67. These clones do not express TAM-67 in the presence of doxycycline, but do express high levels of TAM-67 protein when doxycycline is removed from the media (Figure 5b).

The functional activity of TAM-67 in these MCF7 clones was determined by analysing inhibition of basal and TPA-induced AP-1 transactivating activity (Figure 5c,d). Cells were transfected with the AP-1 reporter plasmid, and then split 1:2. Half the cells were cultured in medium containing doxycycline while the other half of the cells were grown in medium without doxycycline. After induction, the cells were harvested for measuring basal AP-1 activity, or were treated with TPA for 4–6 h and then harvested to measure TPA-induced AP-1 activity. Figure 5c shows the results of transcriptional activation assays of basal AP-1 activity in the present of doxycycline (TAM-67 not expressed), or in the absence of doxycycline (TAM-67 expressed). Removal of doxycycline did not affect basal AP-1 activity in vector-transfected clones (Clones #1 and #7), but did significantly reduce basal AP-1 activity in both the TAM-67 expressing clones (Clones #62 and #67) (Figure 5c).

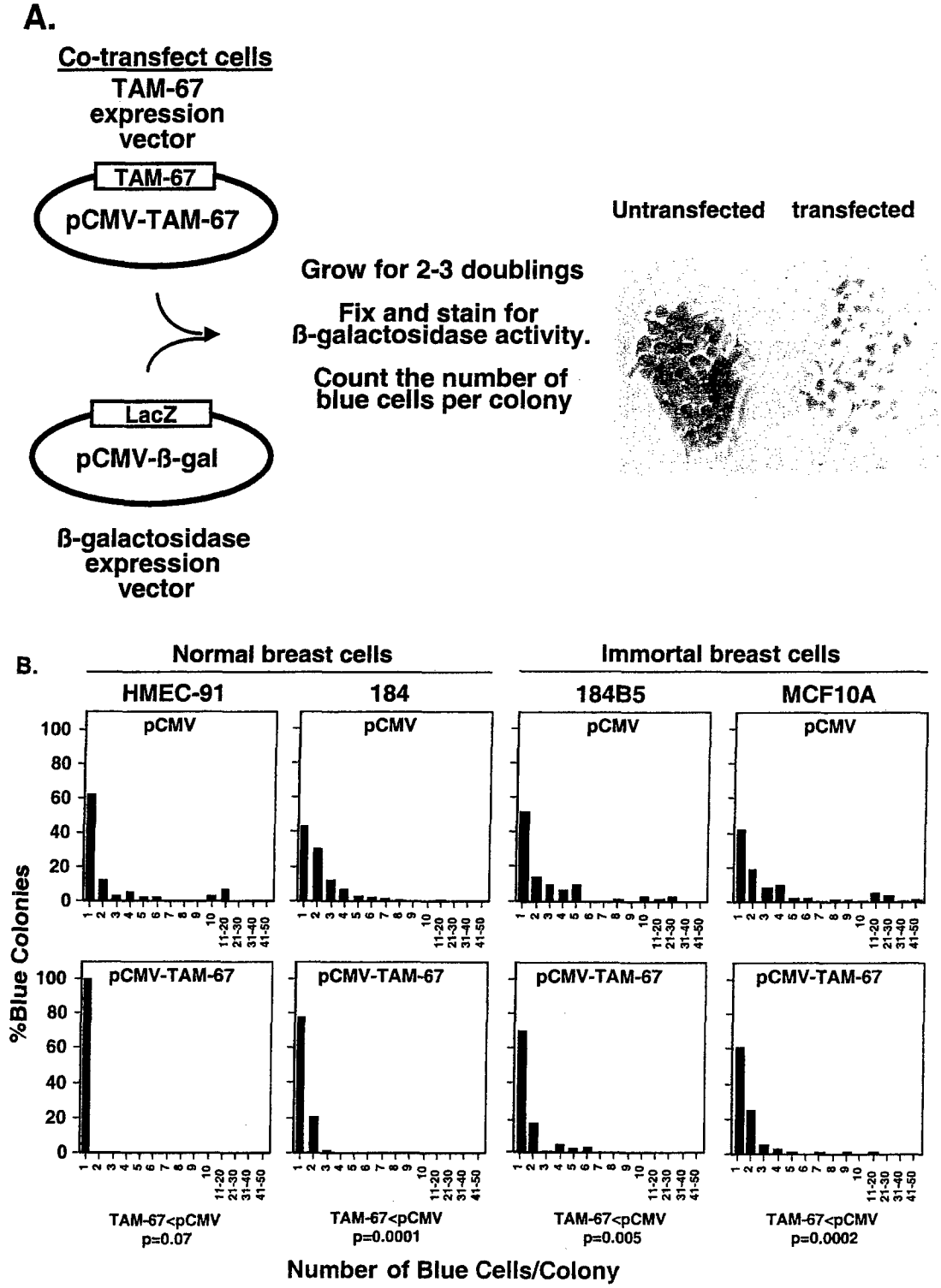

Figure 4 Single cell proliferation assay of normal and immortal breast cells. (**a**) Schematic diagram of the single cell proliferation assay. MCF7 cells were co-transfected with pCMV (5 μg) and pCMV-β-gal (0.5 μg) and stained with X-gal. A transfected colony (blue cells) and an untransfected colony (white cells) are shown. (**b**) Single cell proliferation assay of normal and immortal breast cells. The indicated breast cells were transfected with pCMV-β-gal (0.5 μg) and either pCMV (5 μg) or pCMV-TAM-67 (5 μg). After approximately three doublings, the transfected cells were identified by staining *in situ* for β-galactosidase activity and the number of transfected cells per colony were counted. The results are shown as histograms of 1 – 20 cells per colony and were analysed using the Wilcoxon rank sums test as described in the Statistical Analysis section of Materials and methods. *P* values from the Wilcoxon rank sums test are shown

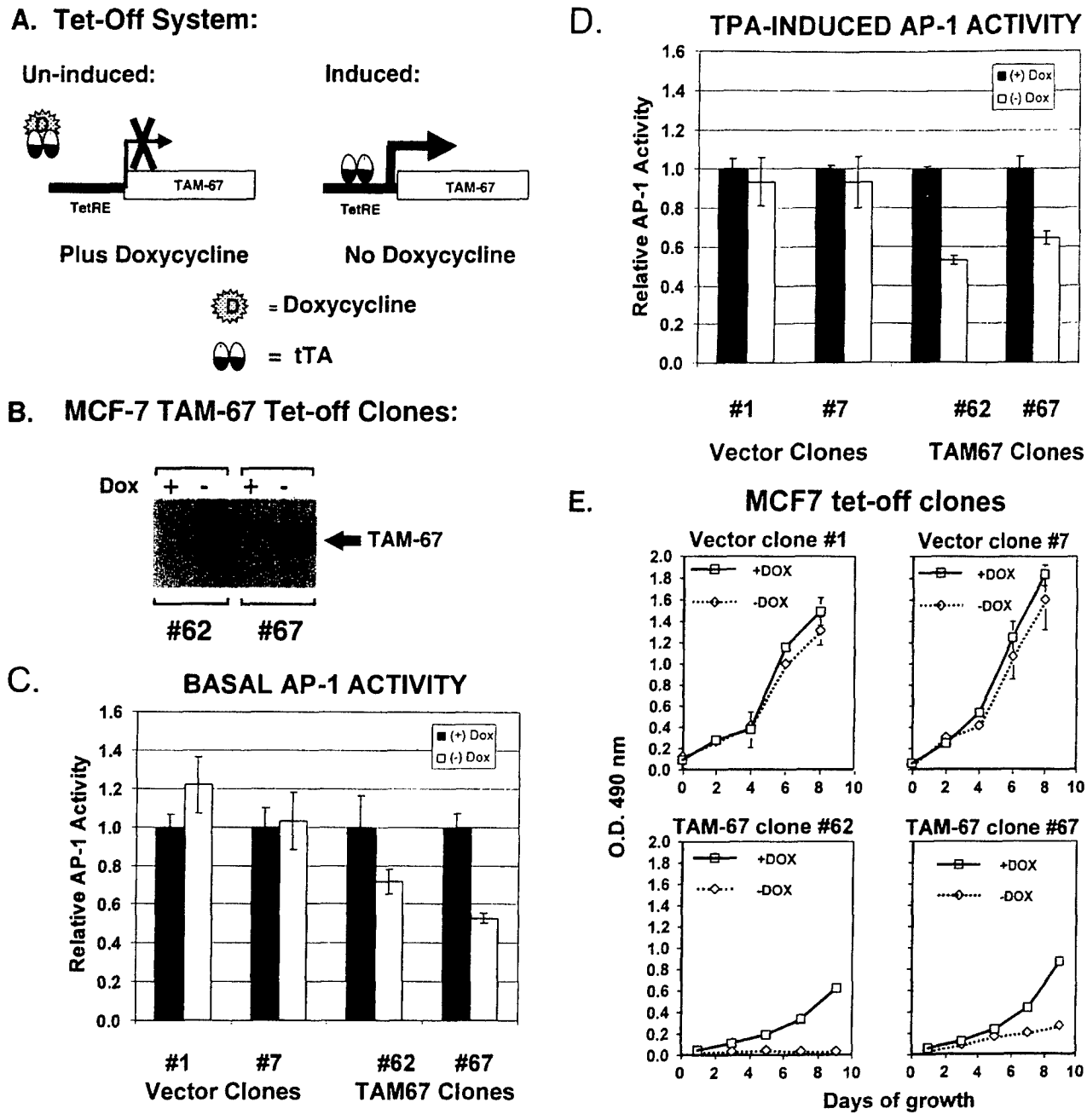

Figure 5 Induction of TAM-67 protein in MCF7 cells. (**a**) The Tet-off inducible protein expression system is shown. The Tet-off system utilizes the tetracycline dependent transcriptional repression activity of the tTA protein. The TAM-67 cDNA was cloned downstream of a tTA repressible CMV promoter element (TetRE) in a plasmid having a hygromycin-selectable marker. With the Tet-off system transfected cells are selected for hygromycin-resistance in the presence of doxycycline to maintain repression of TAM-67 expression and protein expression is induced by removal of doxycycline. (**b**) TAM67 protein expression in MCF7 Tet-off TAM-67 clones. Immunodetection of induced TAM-67 protein expression in MCF7 Tet-off cells. Total cellular protein was extracted 48 h after growing the cells in the presence (+) or absence (−) of doxycycline, and equal amounts of protein was analysed for TAM-67 expression using anti-cJun antibody. The TAM-67 protein band is indicated with an arrow. (**c**) Inhibition of basal AP-1 activity in MCF7 Tet-off TAM-67 clones. Basal AP-1 activity was determined by measuring the effect of TAM-67 protein induction on the activity of the transfected AP-1-dependent luciferase reporter plasmid, Col-Z-Luc. Cells were transfected with 1 μg of Col-Z-Luc (and 0.5 μg of CMV-βGal plasmid to measure transfection efficiency) for 12 h and split into two plates, with or without doxycycline. After 36 h of incubation, the cells were harvested to measure basal AP-1 activity. Cell extracts were prepared, and luciferase and β-galactosidase activity were measured as described in Materials and methods. Each transfection was done in triplicate, and the luciferase results were normalized for differences in transfection efficiency using the β-galactosidase results. The data are presented as the luciferase activity in the presence of TAM-67 relative to the luciferase activity in the absence of TAM-67 for each MCF7 clone. Mean values are shown and error bars show standard deviation from the mean. (**d**) Inhibition of TPA-induced AP-1 activity in MCF7 Tet-off TAM-67 Clones. The effect of TAM-67 on TPA-induced AP-1 activity was determined by measuring AP-1 activity in the presence or absence of doxycycline in clones transfected with the AP-1 dependent luciferase reporter plasmid, Col-Z-Luc. The cells were transfected with 1 μg of Col-Z-Luc Luc (and 0.5 μg of CMV-βGal plasmid to measure transfection efficiency) for 12 h and split into two plates, with or without doxycycline. After 36 h of incubation the cells were treated

Figure 5d shows the results of experiments measuring TPA-induced AP-1 activity in MCF7 cells. When the cells were grown in the presence of doxycycline (uninduced conditions), AP-1 activity was induced more than fivefold with TPA treatment (data not shown). TPA-induced AP-1 activity was not affected by removal of doxycycline in the MCF7 vector clones (Clones #1 and #7, Figure 5d). In contrast, when TAM-67 transfected cells were grown in the absence of doxycycline (TAM-67 induced), TPA-induced AP-1 activity was reduced in both MCF7-TAM67 clones (Clones #62, and #67, Figure 5d). These results demonstrate that TAM-67 protein inhibits both basal and TPA-induced AP-1 activity in these inducible MCF7 breast cancer cell lines.

We next directly measured the growth of the MCF7 cells under conditions in which TAM-67 was either repressed or induced (Figures 5e). As shown in Figure 5e, the growth of both of the MCF7 Tet-off TAM-67 clones was inhibited when doxycycline was removed from the media. The growth of two independently isolated vector-transfected clones was not inhibited by withdrawal of doxycycline. These results, along with the results of studies of transiently-transfected cells, shown in Figure 3, demonstrate that the growth of MCF7 cells is inhibited by the expression of the AP-1 inhibitor, TAM-67.

Discussion

The above results show that the growth of normal and immortal human mammary epithelial cells and MCF7 breast cancer cells is inhibited by AP-1 blockade. We have previously shown that normal human breast cells express high basal levels of AP-1 activity and that breast cancer cells express lower levels of AP-1 activity (Smith *et al.*, 1997). The studies reported here suggest that the high levels of AP-1 activity in normal and malignant breast cells reflect these cells' dependence on AP-1 for their growth. In addition, the present results show that some breast cancer cells, but not all, also depend on AP-1 for the growth.

The present results demonstrating that premalignant breast cells depend on AP-1 to transduce mitogenic signals is consistent with previous reports demonstrating that normal human mammary epithelial cells require peptide growth factors to support their growth. Stampfer and Yaswen (1992) and Zajchowski *et al.* (1988) have demonstrated that peptide growth factors are required for the *in vitro* growth of normal human mammary epithelial cells. In addition, Stampfer *et al.* (1993) have shown that 184 normal human mammary epithelial cells, and the immortalized derivative 184B5, are both dependent on TGFα for continued growth demonstrating that normal breast cells require peptide growth factors to sustain their growth. The present results extend this observation to show that the activity of the AP-1 transcription factor, a downstream transducer of these peptide growth factors, is critical for the growth of normal and immortal breast cells.

Our results also demonstrate that certain breast cancer cells are less dependent on AP-1 for their growth than are normal breast cells. The breast cancer cell line most sensitive to AP-1 inhibition was MCF7. The other cancer cell lines studied (T47D, MDA MB 231 and MDA MB 435) were resistant to AP-1 blockade. These results suggest that at least for some breast cancer cell lines (T47D, MDA MB 231 and MDA MB 435), activation of AP-1-dependent pathways may not be essential for their growth. The breast cancer cell line T47D was observed to have an increased colony forming efficiency when AP-1 activity was blocked. This observation indicates that AP-1 activity may be involved in negative growth regulation of this breast cancer cell line. Studies are ongoing to investigate the role of AP-1 in negative growth regulation of T47D breast cancer cells.

We are currently investigating the mechanism by which AP-1 blockade leads to growth suppression of breast cells. Depending on cell type, AP-1 transcription factors transduce mitogenic signals from peptide growth factors, or stress signals through the SAPK or JNK pathways (reviewed in Karin *et al.*, 1997). Thus, AP-1 blockade could inhibit growth either by blocking cell cycle progression, or by inducing apoptosis. Preliminary results from our laboratory suggest that TAM-67 inhibits cell proliferation in MCF7 cells by suppressing entry into the S phase of the cell cycle, without inducing apoptosis (data not shown). This interference with S phase entry is consistent with previous studies of the role of AP-1 in rat fibroblasts. Kovary and Bravo (1991) have previously shown in fibroblasts that microinjection of antibodies specific for Jun or Fos proteins inhibits cell cycle progression and entry into S phase. Based on our preliminary studies, we predict that AP-1 blockade induced by the cJun dominant-negative, TAM-67, also blocks cell cycle progression and entry into S phase in human breast cells.

It is possible that breast cancer cells that have genetic alterations in tumor suppressor genes, such as p53 or Rb mutations, or overexpression of oncogenes, such as c-*erb*B2/her2/*neu*, c-*myc*, or cyclin D, no longer

with TPA for 4–6 h, after which time cell extracts were made and luciferase and β-galactosidase activity was measured. Each transfection was done in triplicate. The data are presented as luciferase activity in the presence of TAM-67 relative to luciferase activity in the absence of TAM-67 for each clone. Mean values are shown and error bars show standard deviation from the mean. (e) Effect of induced TAM-67 expression on the growth of MCF7 and MDA MB 435 breast cancer cells. Growth of MCF7 Tet-off vector and TAM-67 clones was determined in the presence (squares) and absence (diamonds) of doxycycline. Cell growth was measured using the MTS assay as described in Materials and methods. Each point represents the mean of three independent cultures, and error bars show standard deviation from the mean

require mitogenic signals that are normally transduced by AP-1. The observation that normal human breast cells, which lack these genetic alterations, require AP-1 activity for their growth is consistent with this hypothesis. Most of the breast cancer cells studied here have known genetic alterations (Bonsing *et al.*, 1997; Katayose *et al.*, 1995; Lesoon-Wood *et al.*, 1995). T47D, MDA MB 231 and MDA MB 435 have known p53 mutations, while MCF7 breast cancer cells, 184 normal breast cells, and the immortalized cells have been shown to express normal p53 (Lehman *et al.*, 1993). These genetic alterations may disrupt the normal mitogenic signal transduction pathway at a step distal to AP-1, and thus render these transformed cells more resistant to AP-1 inhibitors.

Recent studies of the expression of Jun and Fos family members in human breast tumors also suggest that these proteins have variable expression in human breast tumors. Bamberger *et al.* (1999) investigated the expression of members of the AP-1 family in 53 breast tumors. They observed relatively uniform expression of cJun, JunB, cFos, and Fra2 and variable levels of JunD and FosB. In this study, the authors noted that the expression of FosB correlated with ER-positivity and a well-differentiated phenotype, while expression of Fra1 showed a strong negative correlation with FosB expression, ER receptor positivity, and the differentiation status. Thus, tumors expressing FosB (the well differentiated, ER-positive tumors) would be expected to be sensitive to AP-1 inhibitors, such as TAM-67. Fra-1 has been shown to inhibit AP-1 transcription factor activity (Yoshioka *et al.*, 1995), and thus tumors expressing Fra-1 may be resistant to AP-1 inhibitors. Our present results support this hypothesis: MDA MB 231 cells, which express high levels of Fra-1, are resistant to the anti-proliferative effects of TAM-67 (see Figure 3), while MCF7 cells, which express FosB, but do not express Fra-1 (Chen *et al.*, 1996, and unpublished observation), are sensitive to TAM-67.

More recent studies performed by Gee *et al.* (2000) examined the activation of the AP-1 transcription factor complex in human breast tumors. They measured the expression of phospho-cJun in 78 primary breast tumors. These authors found that phospho-cJun expression was associated with expression of peptide growth factors and their receptors (TGFα and EGFR), as well as with expression of the phosphorylated form of the activating kinase of cJun, JNK. They observed correlations between high phospho-cJun expression and decreased overall survival and presence of distant metastasis. In addition, estrogen receptor-positive tumors that expressed high phosph-cJun developed progressive disease more rapidly than did tumors expressing low phospho-cJun. These clinical data suggest that in a subset of breast tumors, the AP-1 transcription factor is activated, and that this transcription factor is likely transducing growth factor signals *in vivo*. These clinical observations also provide rationale to target this transcription factor for the prevention and treatment of breast cancer.

The present results and those of Gee *et al.* (2000) and Bamberger *et al.* (1999) demonstrate that the AP-1 transcription factor is an important mitogenic signaling complex for normal and malignant breast cancer cells. These studies suggest that this transcription factor complex could be targeted for the development of future therapeutic agents. Thus, agents that inhibit AP-1 or that block AP-1 activation, such as inhibitors of Jun N-terminal kinases, may be useful agents for the prevention or treatment of breast cancer.

Materials and methods

Primary cell cultures and cell lines

The human mammary epithelial cells and cell lines used in these studies are described in previous studies by Smith *et al.* (1997). Cells used include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9–10); normal 184 cells provided by Dr Martha Stampfer (Stampfer and Yaswen, 1992); nontumorigenic immortal cell lines derived from benzo(a)pyrene-treated 184 cells 184A1 and 184B5 (Stampfer and Bartley, 1985), a nontumorigenic spontaneously immortalized HMEC cell line MCF10A (from Dr J Russo); and cancer cells: MCF7, a human breast adenocarcinoma cell line provided by Dr Ken Cowan, and T47D, MDA MB 231, and MDA MB 435 (from ATCC). Cells were grown in the following culture media: MEGM (Clonetics, San Diego, CA, USA) for normal HMECs 184, 184A1, and 184B5 (Stampfer *et al.*, 1980; Stampfer and Bartley, 1985) DME/F-12 with 5% horse serum and supplements for MCF10A (Ciardiello *et al.*, 1990; Soule *et al.*, 1990) and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for the breast cancer cell lines. MCF-7 tTA cells were purchased from Clontech.

Transfection of breast cells

The breast cells 184, HMEC, 184B5, MDA MB 231, MCF7, and T47D were transfected using Fugene 6 reagent (Boehringer–Mannheim); MCF10A and MDA MB 435 breast cells were transfected using the LT-1 transfection reagent (PanVera Corp.) according to manufacturer's recommendations.

Western analysis

Whole cell protein extracts normalized were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). Primary antibody used was rabbit anti-cJun Ab-1 from Oncogene Science (Cambridge, MA, USA). Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

Luciferase assay to measure AP-1 activity

AP-1 transcriptional activating activity in cells was measured using the enhanced luciferase assay (Tropix) as previously described (Chen *et al.*, 1996). The cells were transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter

which contains a single AP-1 binding site (TGAG/CTCA) between nucleotides −73 and −63. Transfected cells were lysed 36 h after transfection and luciferase activity was measured with equal amounts of cell extract. The cells were also transfected with a CMV-βGal plasmid and β-galactosidase activity was measured to normalize the luciferase results for transfection efficiency as previously described (Chen *et al.*, 1996).

Cell growth assays

Colony formation assay Two × 10^5 cells were co-transfected in 35 mm wells with 0.5 μg pZeoSV (Invitrogen), and 5 μg of either pCMV (empty vector) or pCMV-TAM-67. Twelve hours after transfection the cells from each 35 mm well were split into four 35 mm wells. Twenty-four hours after the Transfection, Zeocin (Invitrogen) was added to a final concentration of 400 μg/ml. All cells were found to be killed at this concentration if not transfected with the Zeocin resistance plasmid. After 2 weeks of selection in Zeocin, resistant colonies were stained with crystal violet and counted.

Single cell proliferation assay Cells were co-transfected as described for the colony forming efficiency assay with 0.5 μg of pCMV-ß-gal and 5 μg of either pCMV (empty vector) or of pCMV-TAM-67. Twelve hours after transfection the cells were trypsinized and replated as single at cells densities of 0.2 to 1.0 × 10^5 in 100 mm plates. After approximately three doublings, colonies of cells were fixed and stained with X-Gal to detect cells expressing ß-galactosidase *in situ*. Colonies containing blue cells were visualized by light microscopy and scored for the number of blue cells per colony. The cells in these blue colonies all received DNA and arose from a single transfected cell. Therefore, transfection efficiency in these counted blue colonies is 100%.

Cell proliferation assay of stably transfected Tet-off cell lines The CellTiter 96™ AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) was used to measure breast cancer cell growth according to the protocol provided by the manufacturer. One thousand to 2000 cells were seeded in a 96 well plate in media containing doxycycline, and the next day doxycycline was removed in half the samples to induce the transfected gene (MCF7 tTA-vector or -TAM67 lines). During the experiment the medium was replaced every other day. A solution containing a 20 : 1 ratio of MTS and PMS was added to the cells for 2 h at 37°C and absorption at 495 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption ± standard error.

Statistical analyses

Colony formation assays result in counts of numbers of colonies. After log-transformation, as indicated by Box–Cox analysis (Box and Cox, 1962), TAM-67 transfected cells were compared to vector alone using two-sample *t*-tests. Single cell proliferation assays results in a distribution of cells per colony for each cell type. Wilcoxon rank sum tests were used to compare distributions between TAM-67 and vector transfected cells.

Abbreviations

AP-1: Activating Protein-1; ER: Estrogen receptor; HMECs: Human mammary epithelial cells; IGF: insulin-like growth factor; SCPA: Single cell proliferation assay; SEM: standard error of the mean; TGFα: transforming growth factor alpha; TPA; 12-O-tetradecanoylphorbol-13-acetate; X-Gal: 5-Bromo-4-Chloro-3-Indoyl-ß-galactopyranoside.

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Breast Cancer Cells Have Lower Activating Protein 1 Transcription Factor Activity than Normal Mammary Epithelial Cells[1]

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ABSTRACT

To determine whether normal breast cells have different levels of activating protein 1 (AP-1) expression and activation relative to breast cancer cells, we have compared the level of c-Jun and c-Fos expression and AP-1 activity in human mammary epithelial cells (HMECs) at different stages of transformation (normal proliferating HMECs, immortal HMECs, oncogene-transformed HMECs, and breast cancer cell lines). These studies demonstrated that normal and immortal HMECs have a high basal level of expression of cJun and cFos and higher AP-1 DNA-binding and transcriptional activating activities than do oncogene-transformed HMECs or human breast cancer cells, with a gradual decrease in AP-1 transactivating activity as cells progress through the carcinogenesis pathway (normal > immortal > oncogene-transformed > cancer cell lines). The AP-1 activity in normal or immortal cells was not modulated by growth factor supplementation or oncogene overexpression, as it is in breast cancer cells. However, the addition of suramin, a nonspecific growth factor antagonist, did inhibit AP-1 in these HMECs, suggesting that this high level of AP-1 present in normal HMECs may be due to autocrine stimulation of growth factor pathways. The differences in AP-1 activity in normal and malignant breast cells may indicate that normal cells are more dependent on AP-1-mediated signals for their growth than are breast cancer cells.

INTRODUCTION

Multiple growth factors have been identified that stimulate the proliferation or differentiation of normal HMECs[4] and breast cancer cells (reviewed in (1–3)). EGF stimulates the growth of normal HMECs (2) as well as breast cancer cells (1, 3), and heregulin has been found to modulate the growth and differentiation of immortal HMECs (4). Other hormones that affect the growth of breast cancer cells include estrogen (5, 6) and insulin-like growth factors (IGF-1 and IGF-2; Ref. 7), which induce proliferation, and retinoids, which inhibit proliferation and induce differentiation (8, 9). Steroid hormones such as estrogen and retinoids directly activate steroid hormone receptor transcription factors, whereas peptide hormones induce the activation of second messengers, which in turn activate nuclear transcription factors (10, 11). The ultimate effect of growth factor stimulation of these different signal transduction pathways is the activation of nuclear transcription factors that regulate transcription of genes. Differential expression of these target genes can determine whether the cells proliferate, differentiate, or become transformed.

The present study characterizes the expression and activity of one such transcription factor, the AP-1 complex in human breast cells, which is activated by the stimulation of mitogenic signal transduction pathways. This transcription factor is a complex of the Jun and Fos proto-oncoproteins (12, 13). AP-1 complexes are formed by dimers of Jun family members (c-Jun, JunB, and JunD) or heterodimers of the Jun family members with the Fos family members (c-Fos, Fos B, Fra-1, and Fra-2). AP-1 complexes bind to a specific target DNA site (also known as the TRE) found in the promoters of several cellular genes such as human collagenase, stromelysin, plasminogen activator (uPA), and plasminogen activator inhibitor (PAI-1) and activate the transcription of these genes (12, 13).

The AP-1 complex either positively or negatively regulates transcription of target genes, depending on the composition of the heterodimers (14–18). In response to different stimuli, such as growth factor stimulation, cellular stress, or even UV light stimulation, the expression and activity of Jun and Fos proteins are rapidly and transiently induced (19–21). In addition, tumor-promoting agents, such as TPA, have also been shown to act via the AP-1 pathway (22, 23). Several other families of transcription factors also interact with Jun and Fos proteins to affect their activity. Other leucine-zipper proteins such as members of the cAMP-responsive element binding protein/activating transcription factor family have been shown to heterodimerize with c-Jun and affect transcriptional activity of the AP-1 complex (24). Cross-coupling between c-Fos and c-Jun and the nuclear factor-κB p65 protein has also been demonstrated and was found to enhance DNA binding and transactivating activity of both nuclear factor-κB and AP-1 (25). In addition, several steroid hormone receptors, such as glucocorticoid receptor, retinoic acid receptors, and estrogen receptor, can enhance or repress AP-1 activity by a mechanism that may involve direct protein-protein interaction, a process termed transcription factor "cross-talk" (reviewed in Ref. 26).

The purpose of the present study is to determine the expression and activity of AP-1 complex in HMECs at different stages of the carcinogenesis pathway. We have shown previously that the AP-1 transcription factor is expressed in breast cancer cells and is activated by serum, TPA, or peptide growth factor stimulation in these cells (27). In this study, we determined whether AP-1 expression or activity of normal, immortal, and oncogene-transformed HMECs changes upon transformation of human breast cells. We measured the basal level and growth factor-induced expression of c-*jun* and c-*fos* genes, AP-1 DNA binding, and transactivating activities in proliferating normal, immortal, oncogene-transformed HMECs, and breast cancer cell lines. We also determined the effect of overexpressing activated oncogenes (c-*erb*B2 and c-Ha-*ras*) on the AP-1 transactivating activity in immortal nontumorigenic HMECs and in tumorigenic breast cancer cell lines. Results from these studies showed that normal HMECs have a higher basal level of AP-1 than do cancer cells, and that the high basal activity in normal and immortal HMECs is not further increased by exogenous growth factors or by the expression of activated onco-

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[4] The abbreviations used are: HMEC, human mammary epithelial cell; EGF, epidermal growth factor; IGF insulin-like growth factor; AP-1, mammalian activating protein-1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA response element; TGF, transforming growth factor; CAT, chloramphenicol acetyltransferase; CMV, cytomegalovirus; βGal, β-galactosidase; ATTC, American Type Tissue Collection.

genes. The characterization of the transcription factors activated by growth factors and oncogenes in HMECs should lead to a more complete understanding of the signal transduction pathways that control proliferation and transformation of breast cells.

MATERIALS AND METHODS

Primary Cell Cultures and Cell Lines

Human mammary epithelial cells and cell lines used in these studies include normal HMECs isolated from epithelial organoids of human breast from Clonetics (passages 9–10); normal 184 cells (2); 184A1 and 184B5, nontumorigenic immortal cell lines derived from benzo(*a*)pyrene-treated 184 cells (28); MCF10A (from Dr. J. Russo), a nontumorigenic spontaneously immortalized HMEC cell line; MCF10AneoT (from Dr. J. Russo, Fox Chase Cancer Center, Philadelphia, PA), a transformed cell line derived from MCF10A transfected by c-Ha-*ras*; MCF7 WT (wild type), a human breast adenocarcinoma cell line; MCF7 Adria, a doxorubicin (Adriamycin)-resistant subclone of MCF7 WT (from Dr. K. Cowan, National Cancer Institute, Bethesda, MD); and SKBr-3 (from American Type Culture Collection, Rockville, MD), a human breast cancer cell line with c-*erb*B2 amplification. Cells were grown in the following culture media: MEGM with or without sodium bicarbonate (Clonetics, San Diego, CA) for normal HMECs, 184, 184A1, and 184B5 (28, 29); DMEM/F-12 with 5% horse serum; and supplements as described (30, 31) for MCF10A and MCF10AneoT [with 400 μg/ml Geneticin (G418), Life Technologies, Inc., Gaithersburg, MD], and Improved MEM (high zinc option; Life Technologies, Inc.) supplemented with 10% FCS and penicillin/streptomycin for MCF7 WT, MCF7 Adria, and SKBr-3.

Growth Factor, Inhibitor, and Antibodies

For growth factor studies, HMECs were grown in minimal media (without growth factor/serum supplements) for at least 15 h and then stimulated using human recombinant EGF (UBI, Lake Placid, NY) at 10 ng/ml at varying times. Suramin was used at a concentration of 1 mg/ml. In experiments using neutralizing antibodies, cells were incubated with monoclonal antibody specific for TGF-α (R & D, Minneapolis, MN; 10 μg/ml), which has previously been shown to inhibit TGF-α-induced proliferation of the four MBr-5 epithelial cell lines (R & D, product insert) or control monoclonal antibody MOPC21 (Sigma, St. Louis, MO) and incubated for 24–48 h, then harvested for CAT assay.

Transient and Stable Transfections of HMECs

HMEC were transfected either by calcium-phosphate precipitation procedure or by Lipofectamine (Life Technologies, Inc.). Using Lipofectamine, cells plated overnight in regular media were washed twice with PBS, and 4 ml of OPTI-MEM (Life Technologies, Inc.) were added. DNA and Lipofectamine reagent (1:4 ratio) were incubated for 30 min at room temperature, added to the cells, and incubated for 6 h. The cells were washed twice with PBS and changed to regular media or serum starved. For stable transfections, cells were cotransfected with either pCOc-*erb*B2A (from T. Yamamoto, University of Tokyo, Tokyo, Japan) or T-24 activated c-Ha-*ras* (from R. Weinberg, Whitehead Institute, Cambridge, MA) and pSV2neo. Two days after transfection, the cells were harvested and grown in G418-containing media (Geneticin; Life Technologies, Inc.; 150 μg G418/ml) for selection of neomycin-resistant colonies. G418-resistant colonies were picked after 2–4 weeks.

RNA Analysis

RNA was harvested from continuously growing cells, cells grown in minimal media, or cells harvested at different time points after addition of EGF, by lysis with guanidinium isothiocyanate, and purification by centrifugation through a cesium gradient. Ten μg of total RNA were then separated using 1% agarose/formaldehyde gel electrophoresis and transferred onto Zetaprobe nylon membranes (Bio-Rad, Richmond, CA). The blots were then hybridized with ^{32}P-labeled c-*jun* or c-*fos* DNA probes, washed with high stringency (0.1× SSC, 0.1% SDS), and exposed to autoradiography.

Measurement of AP-1 DNA-binding Activity

Preparation of Nuclear Extracts. Nuclear extracts were prepared as described previously (27). Briefly, cells were plated in 100-mm culture dishes and grown overnight. The media was not changed for 48 h for continuously growing cells. For EGF induction, the cells were grown in minimal media for at least 15 h, and then EGF was added. The cells were then harvested at various times, washed with PBS, and lysed in cell lysis buffer (with NP40). Intact nuclei were isolated by high speed centrifugation and resuspended in nuclear suspension buffer. The nuclei were then lysed by freeze/thawing, and the protein concentration in the resulting nuclear lysate was determined by a colorimetric assay (Bio-Rad).

DNA Binding Assay. The DNA binding assay was performed using the Gel Shift kit (Stratagene, San Diego, CA). Briefly, 5 μg of nuclear protein were mixed with DNA binding buffer and a ^{32}P-labeled double-stranded oligonucleotide containing a single AP-1 consensus binding site (Stratagene) and incubated at room temperature for 30 min. To inhibit specific AP-1 binding, an excess of nonradiolabeled double-stranded AP-1 oligonucleotide (Promega Corp., Madison, WI) was also mixed with some control samples. Bromphenol blue dye was then added to the samples, which were then loaded onto a nondenaturing acrylamide gel to separate protein-bound DNA from free oligonucleotide. The gels were run at 4°C and then dried and exposed to X-ray film or analyzed using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) to quantitate the intensity of retarded bands.

Western Analysis

Equal amounts of protein from nuclear fractions were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad). The blots were blocked using nonfat dry milk in PBS-Tween 20 (0.05%; PBS-T) for 1 h at room temperature or overnight at 4°C, washed with PBS-T, and then incubated with primary antibody [rabbit anti-c-Jun Ab-1 from Oncogene Science (Cambridge, MA), rabbit anti-cFos Ab SC-52 (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-human activated c-*erb*B2 (UBI)] at a 1:250 dilution for 1 h at room temperature. Blots were washed extensively with PBS-T and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Amersham Corp.) for 1 h. Blots were washed with PBS-T and then developed using the enhanced chemiluminescence (ECL) procedure (Amersham).

CAT Assay to Measure AP-1 Activity

AP-1 transcriptional activating activity in cells was measured using a CAT assay as described previously (27). The cells were transfected using calcium phosphate-mediated or Lipofectamine (Life Technologies, Inc./BRL)-mediated transfection with a reporter gene containing the *CAT* gene linked to a promoter that contains an AP-1 binding site (TGAG/CTCA). The level of expression of this AP-1-dependent *CAT* reporter gene is defined as AP-1 transactivating activity. The CAT reporter DNA was cotransfected with 5 μg of CMV-βGal DNA (Clontech, Palo Alto, CA). The cells were harvested and lysed, and transfection efficiency was determined by measuring β-galactosidase activity in cell lysates. After normalizing for transfection efficiency, CAT activity was measured using TLC and quantitated using a PhosphorImager (Molecular Dynamics). The various CAT reporter plasmids (from Dr. M. Karin) used were: −73/+63 ColCAT, which contains a portion of the human collagenase gene promoter with a single AP-1 site; −60/+63 ColCAT, containing a portion of the human collagenase gene promoter without the AP-1 site (negative control); 1× TRECAT, containing a single copy of a synthetic AP-1 site; 5× TRECAT, containing five copies of synthetic AP-1 site; and ΔtkCAT, containing the thymidine kinase promoter without AP-1 site.

EGF Receptor Quantitation

Cells were plated in 24-well plates and grown until confluent. The medium was aspirated, and cells were repeatedly washed with HITES media (no serum; Life Technologies, Inc.). Varying concentrations of EGF and ^{125}I-labeled EGF (DuPont NEN, Boston, MA) were added sequentially and incubated at 37°C for 30 min. Excess EGF was washed off, and bound ^{125}I was counted using an LKB gamma counter. EGF receptor levels were calculated using Scatchard plot analysis.

RESULTS

Human Mammary Epithelial Cells. To determine the relative expression and activity of the AP-1 complex in normal, immortal, and malignantly transformed HMECs *in vitro,* we studied a series of cells at different stages of transformation. The following cell lines were used: normal HMEC strains (184 and NHMEC); immortal HMEC lines (184A1, 184B5, and MCF10A); oncogene-transformed HMECs (MCF10AneoT, MCF10Ac-Ha-ras3, and 184B5c-*erb*B2A9); and breast cancer cell lines (MCF7 WT MCF7 Adria and SKBr-3; Table 1). This panel of cells includes two independently isolated samples of normal mammary epithelial cells (NHMECs and 184 cells) derived from reduction mammoplasty tissue. The 184 cells have been shown previously to have a normal karyotype and to express markers typical of normal breast epithelial cells *in vivo,* including the luminal markers cytokeratin 18 and polymorphic epithelial mucins (28–30). These normal HMECs have a finite life span and do not display anchorage-independent growth properties.

We also studied three immortal HMEC lines, 184A1, 184B5, and MCF10A. The 184A1 and 184B5 cells were isolated after exposing the normal parental 184 cells to the carcinogen benzo(*a*)pyrene (28). The MCF10A cell line is an independently isolated immortal line that arose spontaneously after isolating mammary epithelial cells from an individual with fibrocystic breast disease (31, 32). These cells have specific karyotypic abnormalities and grow indefinitely in culture. However, they do not show characteristics of fully transformed malignant breast cells, because they retain growth factor dependence, do not display anchorage-independent growth, and do not form tumors in nude mice (28, 31).

The 184B5 and MCF10A immortal cell lines have been transformed with oncogenes to produce cells that will display anchorage-independent growth and tumorigenicity in nude mice. The MCF10AneoT and the MCF10Ac-Ha-ras3 cell lines are two independently transformed MCF10A clones that were produced by transfecting the MCF10A cell line with the c-Ha-*ras* oncogene (33). These cell lines show vigorous anchorage-independent growth, and the MCF10AneoT line (but not the MCF10Ac-Ha-ras3 line) has been shown to be tumorigenic in mice (33). We have also transformed the 184B5 cell line by stably transfecting activated c-*erb*B2. The resulting transformed clonogenic cell line, 184B5c-*erb*B2A9, displays anchorage-independent growth but does not form tumors in nude mice (data not shown).

We also compared the AP-1 activity of these HMECs with two breast cancer cell lines, wild-type MCF7 (Cowan strain; MCF7 WT) and an Adriamycin-resistant subclone of MCF7 WT, MCF7 Adria. We chose to use these two MCF7 cell lines to compare with normal cells, because in a previous study of AP-1 activity in multiple human breast cancer cell lines, we found that of all cell lines studied, MCF7 WT had the lowest and MCF7 Adria had the highest AP-1 activity in breast cancer cells (as defined by both AP-1 DNA-binding activity and by AP-1 transactivation activity; Ref. 27). The previous study also showed variable levels of AP-1 activity, independent of estrogen receptor level (27). Both the MCF7 WT and MCF7 Adria cell lines show anchorage-independent growth and form tumors in nude mice with estrogen supplement.

Table 1 *Cells and cell lines used in this study*

Cells	Source	Phenotype
Normal HMECs		
NHMEC	Clonetics	Senescent, anchorage-dependent
184	M. Stampfer	
Immortalized HMECs		
184A1	M. Stampfer	Immortal, anchorage-dependent
184B5	M. Stampfer	
MCF10A	J. Russo	
Oncogene-transformed HMECs		
184B5c-erbB2A9	P. Brown	Anchorage-independent, nontumorigenic
MCF10c-Ha-ras3	P. Brown	Anchorage-independent, nontumorigenic
MCF10AneoT	J. Russo	Anchorage-independent, tumorigenic
Breast cancer cell lines		
MCF7WT	K. Cowan	Anchorage-independent, tumorigenic
MCF7Adria	K. Cowan	Anchorage-independent, tumorigenic
SKBr-3	ATTC	Anchorage-independent, tumorigenic

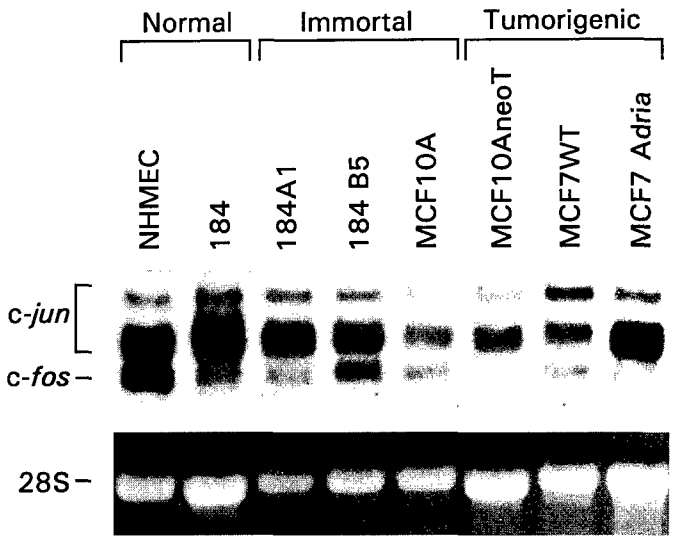

Fig. 1. c-*jun* and c-*fos* RNA expression in normal, immortal, and tumorigenic HMECs. Total RNA was harvested from continuously growing cells, and levels of c-*jun* and c-*fos* mRNA expression were analyzed by Northern blot hybridization using ^{32}P-labeled cDNA probes. The relative amount of RNA loaded in each lane is shown by ethidium bromide staining of rRNA (*28S*).

AP-1 Expression and Activity in HMECs. To study the AP-1 activity in HMECs, we measured the expression of c-*jun* and c-*fos* at the RNA, protein, and functional level in exponentially growing cells. The basal level of mRNA expression of c-*jun* and c-*fos* was determined by Northern blot analysis of RNA samples from normal, immortal, oncogene-transformed HMECs, and breast cancer cell lines that were collected during exponential growth. Although these cells grow in different media, the normal, immortal, and oncogene-transformed HMECs had similar growth rates under these conditions. The blot was hybridized to c-*jun* and c-*fos* probes, and the results are shown in Fig. 1. As observed, the cells studied express varying levels of c-*jun*. Normal HMECs have the highest c-*jun* expression, even higher than MCF7 Adria, which had been found to have the highest expression of c-*jun* of all cancer cells in a previous study (27). All normal and immortal HMECs express c-*fos* RNA, whereas in most cancer cell lines (27) and in MCF10AneoT and MCF7 Adria, c-*fos* RNA is undetected by Northern analysis. The very high level of c-*fos* RNA expressed in continuously growing normal cells is striking because c-*fos* expression is typically an immediate-early and transient response to growth factor stimulation and is often not detected by Northern analysis in exponentially growing cancer cells.

We next determined if c-*jun* and c-*fos* RNA expression correlates with the basal protein expression in exponentially growing breast cells. For these experiments, the cells were harvested 48 h after being plated in fresh media. Western analyses of nuclear extracts from these cells using antibodies specific for c-Jun and c-Fos (Fig. 2*A*) demonstrated that the basal levels of proteins in these cells correlate with RNA expression. All of the cells express c-Jun protein at varying levels. c-Fos is highly expressed in all normal and immortal HMECs; however, only one of three tumorigenic cell lines express detectable amounts of c-Fos protein during exponential growth. In general, the high level of c-Fos RNA present in the normal and immortal HMECs

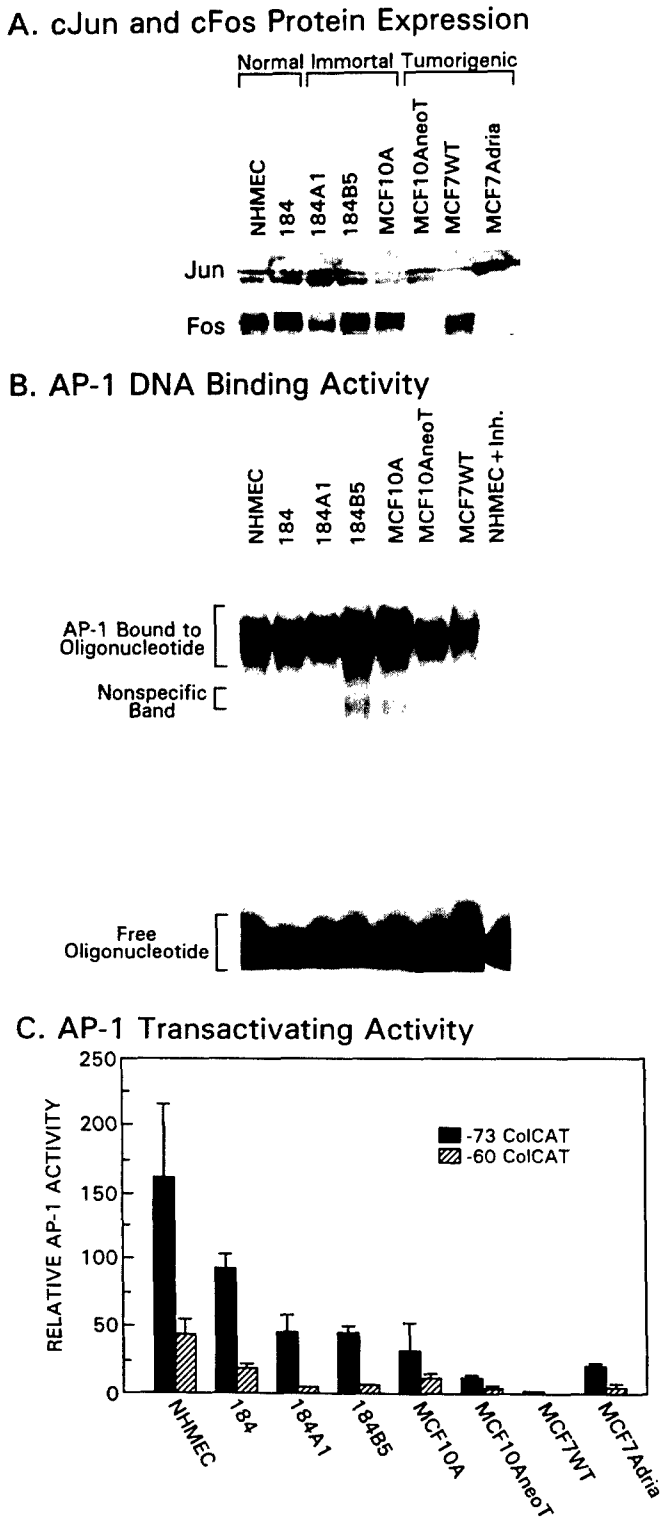

Fig. 2. AP-1 expression and activity in HMECs. *A*, c-Jun and c-Fos protein expression by Western blot analysis. Equal amounts of nuclear extracts from continuously growing cells were electrophoresed on 12% Tris-glycine gel and blotted onto nitrocellulose membrane. For detection of c-Jun and c-Fos proteins, polyclonal anti-c-Jun Ab (Ab-1) and anti-c-Fos Ab (SC-52) were used, respectively, and stained with the ECL detection kit. *B*, AP-1 DNA-binding activity in normal, immortal, and tumorigenic HMECs. DNA-binding activity was determined using a gel shift assay as described in "Materials and Methods." Continuously growing cells were harvested and used for nuclear extract preparation. Relative DNA-binding activity was quantitated using a PhosphorImager. *Inh.*, inhibitor (an unlabeled AP-1 oligonucleotide). *C*, AP-1 transactivating activity in normal, immortalized, and tumorigenic HMECs. AP-1 activity is defined as the difference between transactivation of the −73 ColCAT reporter gene (which contains a single AP-1 responsive site) and transactivation of the −60 ColCAT reporter gene (in which the AP-1 responsive site is deleted). CAT activity was measured as described in "Materials and Methods."

correlates with a high level of c-Fos protein in these cells; however the NHMEC, had higher c-Fos RNA expression than did the normal 184 cells, whereas the Western analysis shows they have similar high c-Fos protein expression. Such differences in RNA expression without major differences in c-Fos protein expression may represent differences in the translation or half-life of c-Fos in the two normal cells.

We then determined the functional AP-1 DNA-binding activity in the different HMECs using the electrophoretic mobility shift assay. To measure AP-1 DNA-binding activity, equal amounts of nuclear protein from exponentially growing cells were incubated with radioactively labeled AP-1 oligonucleotides. The results are shown in Fig. 2*B*. Normal and immortal HMECs have high basal AP-1 DNA-binding activity, whereas the oncogene-transformed cell line, MCF10AneoT, and the breast cancer cell line MCF7 WT have lower AP-1 DNA-binding activity. The normal HMECs have even more AP-1 DNA-binding activity than MCF7 Adria cells (data not shown), which have been shown previously to express very high AP-1 DNA-binding activity compared to other breast cancer cell lines (27).

To test whether the expressed AP-1 proteins can activate transcription, we performed CAT assays to measure AP-1-dependent transactivating activity. Using the −73ColCAT reporter, which has a portion of the collagenase promoter containing an AP-1 site linked to the thymidine kinase promoter, we determined the basal AP-1 transactivating activities in the panel of HMECs. The results were controlled for differences in transfection efficiencies between the cell lines by cotransfecting a βGal expression vector and normalizing for βGal activity. The results of these studies are shown in Fig. 2*C* and demonstrate that proliferating normal, immortal, and malignantly transformed HMECs differ in the level of AP-1 activity. There is a progressive decrease in AP-1 transactivating activity, with the normal HMECs having the highest basal AP-1 transactivating activity, immortal cells having less, and malignantly transformed cells having the lowest AP-1 activity. These data correlate with the AP-1 DNA-binding data shown in Fig. 2*B*. However, the immortal cells show high DNA-binding activity, whereas their AP-1 transactivity activity is less than that in normal HMECs. Such results suggest that the immortal cells express Jun and Fos DNA-binding proteins that do not activate transcription.

Induction of AP-1 Expression and Activity by EGF. To compare the ability of normal, immortal, and oncogene-transformed HMECs and breast cancer cell lines to activate AP-1 in response to exogenous growth factor stimulation, we measured EGF-induced c-*jun* and c-*fos* expression and AP-1 activity in the different breast cells. The presence of EGF receptors in these cells was first verified and quantitated by receptor binding assay using Scatchard analysis. As shown in Table 2, EGF receptor levels vary from 2.3×10^4 (MCF10AneoT) to 1×10^6

Table 2 *Quantitation of EGF receptor in normal, immortal, and tumorigenic HMECs*

Multipoint receptor binding assay using ^{125}I-labeled EGF was performed as described in "Materials and Methods."

Cells/Cell line	No. of EGF receptor molecules/cell ($\times 10^{-5}$)
Normal	
NHMEC	2.70
184	0.75
Immortalized	
184A1	2.00
184B5	2.10
MCF10A	10.00
Oncogene-transformed	
184B5c-*erb*B2	3.00
MCF10AneoT	0.23
Cancer cell lines	
MCF7 WT	0.95
MCF7 Adria	0.96

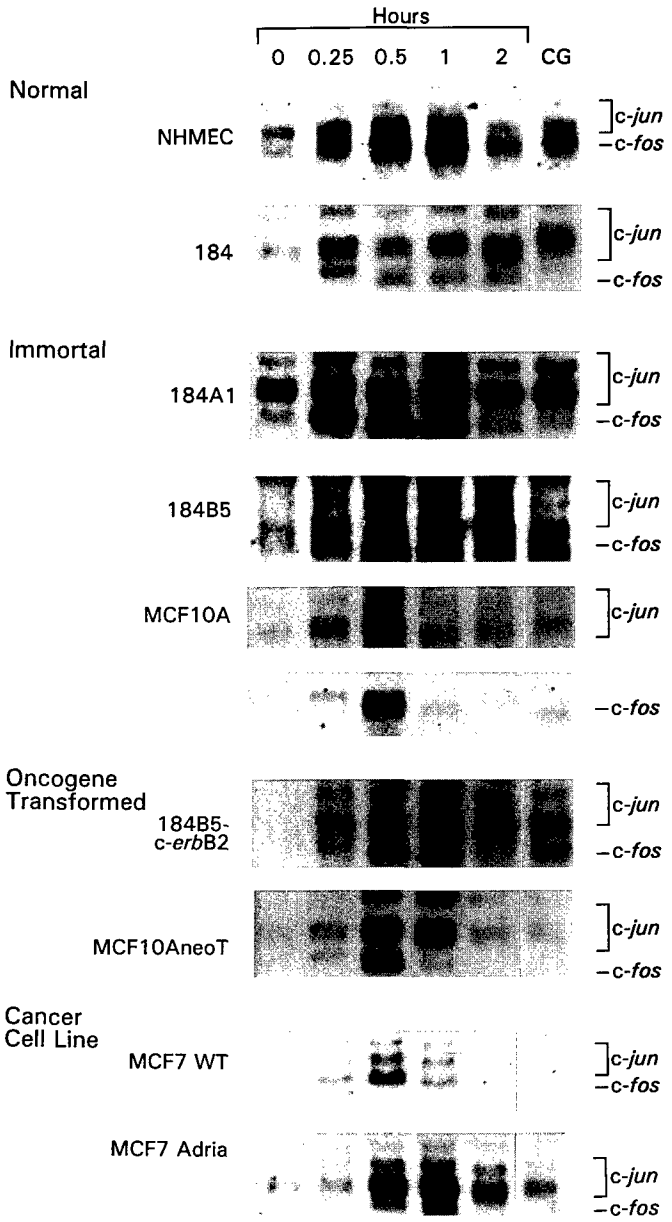

Fig. 3. Effect of EGF on c-*jun* and c-*fos* RNA expression in HMECs. The induction of c-*jun* and c-*fos* RNA by EGF was determined by Northern blot analysis using RNA isolated from HMECs grown in minimal media for 15 h, followed by treatment with EGF (10 ng/ml) for varying times. Similar amounts of RNA were loaded in each lane, as shown by ethidium bromide staining (data not shown). *CG*, continuously growing cells.

(MCF10A) EGFR molecules/cell, with the tumorigenic cell lines having fewer receptors compared to immortal cells. Normal HMECs range from 7.5×10^4 to 2.7×10^5 EGF receptors/cell. These results are comparable to previous studies of EGFR levels in HMECs (34, 35).

We next measured the c-*jun* and c-*fos* RNA expression and AP-1 activity in EGF-supplemented HMECs and breast cancer cell lines. Fig. 3 shows the results of Northern blot analysis of these EGF-supplemented cells. This figure demonstrates that EGF supplementation causes rapid and transient induction of both c-*jun* and c-*fos* RNA in all of the cells studied. These results also show that all of the HMECs can increase c-*fos* and c-*jun* expression in response to EGF at the transcriptional level.

Although EGF induces c-*jun* and c-*fos* mRNA expression in all of the breast cells, the addition of EGF failed to increase AP-1 DNA binding in normal and immortal HMECs. To measure EGF-induced AP-1 DNA-binding activity, we prepared nuclear extracts from normal, immortal, and oncogene-transformed HMECs and breast cancer cell lines that were stimulated by the addition of EGF for increasing times. Fig. 4 shows the results of these DNA-binding experiments and demonstrates that there is only a minimal decrease in AP-1 DNA-binding activity in the normal and immortal cells grown in media without growth factor supplementation. In addition, there was minimal induction of AP-1 DNA-binding activity after EGF stimulation in these nonmalignant cells. These results are in contrast to the strong induction of AP-1 activity in the tumorigenic MCF7 WT. Densitometric analyses of the retarded bands for specific AP-1 binding showed a maximum 6-fold increase over the 0 time point for MCF7 WT and a 2-fold increase for MCF10AneoT, whereas normal and immortal HMECs only had a 1.4-fold increase over the 0 time point. Western blot analysis showed that c-Jun and c-Fos proteins were present in the nuclear extracts, even after 24 h in minimal media (without growth factor supplementation), which may account for the high AP-1 DNA-binding activity observed in these cells (data not shown). It is also likely that autocrine growth factors are secreted by the HMECs under minimal media conditions, which induce high AP-1

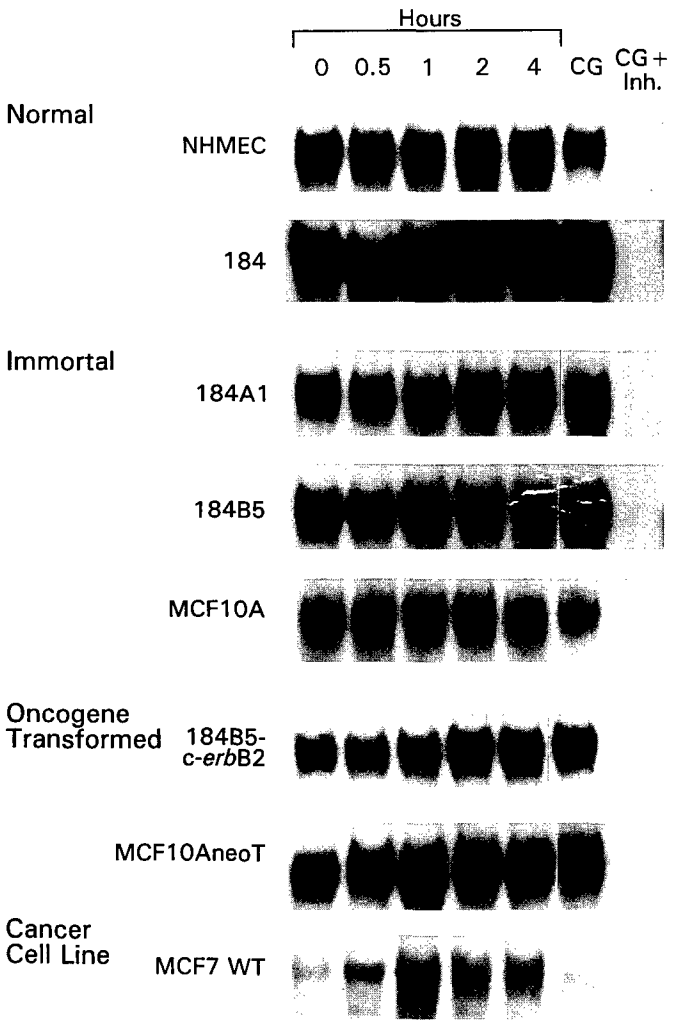

Fig. 4. Effect of EGF on AP-1 DNA-binding activity in HMECs. EGF-induced AP-1 DNA-binding activity was measured in HMECs grown in minimal media for 15 h, followed by treatment with EGF (10 ng/ml) for varying times. The cells were then harvested, and DNA-binding assay was performed as described in "Materials and Methods." Relative DNA-binding activity was quantitated using a PhosphorImager/densitometer. *CG*, continuously growing cells; *Inh.*, inhibitor (an unlabeled AP-1 oligonucleotide).

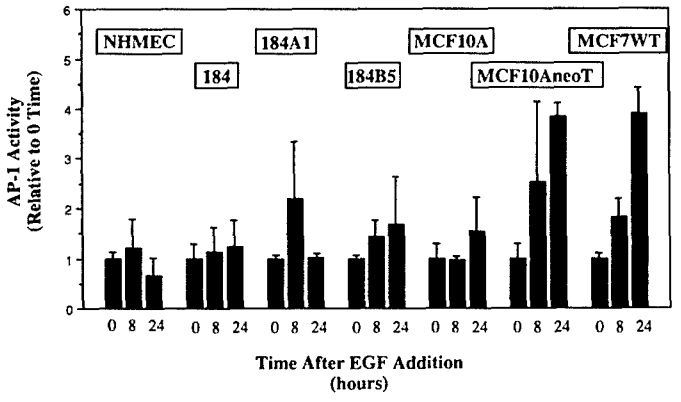

Fig. 5. Effect of EGF on AP-1 transactivating activity in HMECs. EGF-induced AP-1 transactivating activity was measured in HMECs grown in minimal media for 15 h, followed by treatment with EGF (10 ng/ml) and harvested at 0, 8, and 24 h. CAT activity was measured as described in "Materials and Methods." Relative AP-1 activity is shown as mean fold increase of CAT activity over 0 time for each cell line studied. *Bars*, SD.

activity. HMECs have previously been shown to have such autocrine loops involving the EGF receptor (34–36).

AP-1 transactivating activity in normal and immortal HMECs was also not increased by EGF, as shown in Fig. 5. In these experiments, the cells were grown in minimal media and then stimulated with EGF (10 ng/ml) for increasing times. AP-1 activity is shown relative to the AP-1 activity in each cell line without EGF supplement (0 time). These results show that under these conditions, there was no significant induction of AP-1 activity by EGF in normal and immortal cells compared to the 4-fold induction over 0 time observed in the malignantly transformed lines MCF10AneoT and MCF7 WT.

These results from Figs. 4 and 5 suggest that the relatively high levels of c-Jun and c-Fos expression, AP-1 DNA-binding, and transactivating activities are not reduced in HMECs cultured in media without growth factor supplementation and also that exogenous growth factor stimulation of normal HMECs does not increase the already high AP-1 activity present in these cells.

Inhibition of AP-1 Activity in Immortal HMECs. Because normal HMECs have been shown to produce endogenous TGF-α (34–36), it is possible that the AP-1 complex is constantly being activated by an autocrine growth factor loop in the absence of exogenous growth factors. Such autocrine stimulation could account for the high AP-1 DNA-binding and transactivating activities observed in normal and immortal HMECs, even when they are deprived of exogenous growth factors. We, therefore, attempted to lower the endogenous AP-1 activity by blocking such autocrine growth factor loops. To address this issue, we first used a nonspecific growth factor antagonist, suramin, to block the basal AP-1 transactivating activity in immortal cells grown without growth factor supplements (Fig. 6). Suramin has been shown to stimulate human breast cancer cell growth at low concentrations and inhibit growth at high concentrations (37). This suramin-induced inhibition of cell proliferation can be abolished by the addition of IGF-1, basic fibroblast growth factor, or even estrogen, confirming its nonspecific action (37). As shown in Fig. 6, AP-1 transactivating activity of 184B5 cells was decreased in the presence of suramin (1 mg/ml) relative to minimal media after 24–72 h. Suramin also inhibits the growth of these HMECs (data not shown). 184B5 has been shown to secrete lower levels of TGF-α compared to the parental 184 cells (2). When neutralizing antibodies specific for TGF-α were added to 184B5 cells, AP-1 transactivating activity was not inhibited, even after 48 h (data not shown), indicating that the increased AP-1 activity is not caused solely by autocrine stimulation by TGF-α via the EGF receptor. Such results are consistent with the studies reported by Stampfer *et al.* (36) in which c-*jun* and c-*fos* RNA expression were not decreased in 184B5 cells by EGF withdrawal or EGF receptor blockade using a blocking antibody, except for a transient decrease in c-*fos* RNA expression at 24 h. The results presented here suggest that growth factors other than TGF-α may be responsible for the high AP-1 activity observed in immortal HMECs.

Effects of Transfection of Activated Oncogenes on AP-1 Activity of HMECs. In other cell types, overexpression of oncogenes such as *jun*, *ras*, or *neu* induces increased activation of AP-1 (38–41). To determine whether overexpression of such oncogenes can increase AP-1 activity in HMECs, we transiently transfected activated oncogenes in immortal HMECs and measured AP-1 activity in these transfected cells. As shown in Fig. 7*A*, transient transfection of c-*jun* and activated c-*erb*B2 and c-Ha-*ras* did not significantly increase AP-1 transactivating activity in immortal HMECs, even with the use of different AP-1 reporter constructs (containing single or multiple copies of the AP-1 site). Transfection with activated c-*erbB2* reduced the basal AP-1 transactivating activity in these cells. This suggests that overexpression of the activated oncogenes c-*erb*B2 and c-Ha-*ras* does not significantly increase the already high AP-1 activity present in HMECs.

Transient transfection of c-*jun* or activated c-Ha-*ras* in MCF7 WT cells induced a 2-fold increase in AP-1 transactivating activity, whereas transient transfection of c-*jun* increased AP-1 activity by 7-fold in another breast cancer cell line, SKBr-3 (Fig. 7*B*). SKBr-3 has been shown to have an intermediate level of AP-1 activity compared to MCF7 WT and MCF7 Adria (27). Transient transfection of activated c-*erb*B2 did not change the AP-1 transactivating activity in either of the cancer cell lines. These results show that AP-1 transactivating activity can be induced by overexpression of c-*jun* in breast cancer cell lines but not in immortal HMECs.

To further study the effects of activated oncogenes on HMECs, we also determined AP-1 activity in HMECs (184B5 and MCF10A) that had been stably transformed with activated c-*erb*B2 and c-Ha-*ras*. Expression of the activated form of either c-*erb*B2 or c-Ha-*ras* in the stable clones was confirmed by Western immunoblotting (c-*erb*B2) or by reverse transcription-PCR assay (c-Ha-*ras*; data not shown). The stable transfectants showed anchorage-independent growth by soft agar cloning; however, they are not fully malignant because they do not form tumors in nude mice (data not shown), except MCF10AneoT, which has been shown previously to be tumorigenic in irradiated nude mice (33). We measured the AP-1 transactivating activity in these oncogene-transformed cells and in the parental cells and found that the AP-1 transactivating activities in oncogene-transfected cells are the same (c-Ha-*ras* 3) or may even be lower (c-*erb*B2A9 and MCF10AneoT) than the parental cells (Fig. 8). Thus, in

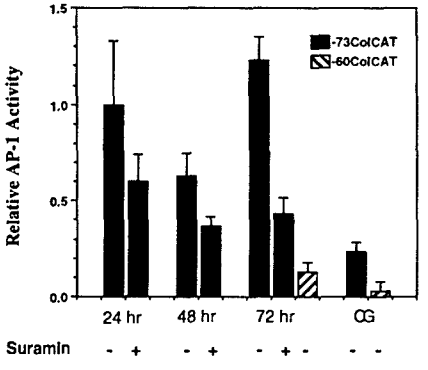

Fig. 6. AP-1 transactivating activity in 184B5 immortal cells treated with suramin. 184B5 cells were cotransfected with AP-1 reporter plasmid (−73ColCAT) and CMV-βGal, grown in minimal media for different time points with or without suramin (1 mg/ml), and harvested for CAT assay. Relative AP-1 transactivating activity is based on 24-h serum deprivation without suramin. *Bars*, SD.

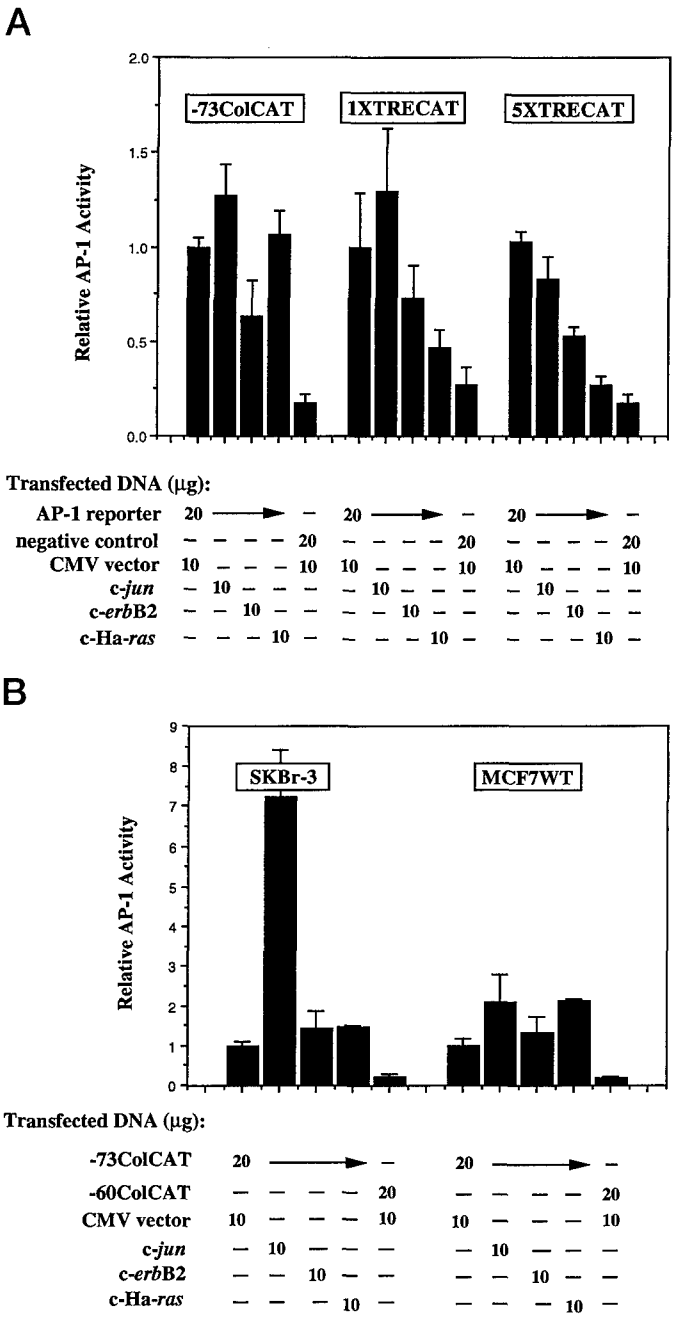

Fig. 7. *A*, AP-1 transactivating activity in 184B5 immortal cells transiently transfected with transforming oncogenes. 184B5 cells were transiently cotransfected with different AP-1 reporter plasmids [−73ColCAT, 1× TRE (containing a single copy of synthetic AP-1 site), and 5× TRE (containing five copies of synthetic AP-1 site)] or their corresponding negative control CAT reporter plasmid plus CMV vector, c-*jun*, activated c-*erb*B2 or c-Ha-*ras*, and CMV-βGal. Cells were harvested after 48 h, and CAT activity was measured as described in "Materials and Methods." Relative AP-1 activity is shown based on CMV vector-transfected as control. *Bars*, SD. *B*, AP-1 transactivating activity in human breast cancer cell lines transiently transfected with transforming oncogenes. SKBr-3 and MCF7 WT cells were transiently cotransfected with c-*jun*, activated c-*erb*B2 or c-Ha-*ras*, −73ColCAT reporter plasmid, and CMV-βGal. Cells were harvested after 48 h, and CAT activity was measured as described in "Materials and Methods." Relative AP-1 activity is shown based on CMV vector-transfected as control. *Bars*, SD.

HMECs that were transiently and stably transfected with oncogenes, AP-1 transactivating activity is not increased from the high levels in the parental, immortal cells.

DISCUSSION

To determine whether changes in AP-1 transcription factor activity occur as HMECs as progress from normal cells with finite life spans to cancer cells, we compared AP-1 expression and activity in two independently isolated normal (nonimmortalized, nontumorigenic) HMECs (NHMEC and 184), three immortal, nontumorigenic cell lines (184A1, 184B5, and MCF10A), oncogene-transformed cell lines (MCF10AneoT and 184B5c-*erb*B2A9), and two human breast adenocarcinoma cell lines derived from MCF7 cells (MCF7 WT and MCF7 Adria). On the basis of the present results, we conclude that proliferating HMECs at different stages in the carcinogenesis pathway display a progressive decrease in AP-1 activity, with normal mammary epithelial cells having the highest AP-1 transactivating activity, immortal cells having intermediate AP-1 activity, and cancer cells having the lowest AP-1 activity. In addition, normal and immortal HMECs do not increase their already high AP-1 DNA-binding and transactivating activities in response to addition of EGF, whereas cancer cells show an increase in AP-1 activity after EGF stimulation.

Oncogene overexpression, which caused malignant transformation of the HMECs, did not increase the AP-1 activity in immortal HMECs and, in some cases, decreased AP-1 transactivating activity. Transfection of activated forms of c-*erb*B2 or c-Ha-*ras* has been shown to activate AP-1 transcriptional activation in other cells, such as rodent fibroblasts. Results from this study suggest that, in HMECs, activated c-*erb*B2 and c-Ha-*ras* may instead be activating other transcription factors that are inhibitory to AP-1 transactivating activity (such as JunB or Fra-1; Refs. 14, 15, and 42).

These studies demonstrate that immortal and malignantly transformed HMECs differ in their ability to modulate AP-1 activity in response to exogenous growth factor and oncogene overexpression. These stimuli, which typically would increase AP-1 activity in fibroblasts (12, 40, 41), do not change the chronically high AP-1 activity in immortal HMECs.

The progressive decline in AP-1 transactivating activity as HMECs become transformed is in contrast to the changes in AP-1 seen in studies of keratinocyte transformation. In previous studies, Dong *et al.* (43) demonstrated that AP-1 activity in keratinocytes increases as they are transformed by TPA or EGF stimulation, and that blockade of AP-1 prevents transformation. In another study of skin carcinogenesis, Domann *et al.* (44) observed a similar increase in AP-1 activity when mouse epidermal cells were transformed by X-irradiation. The results from these studies in keratinocytes and the present studies in human breast cells point out that common transcription factors such as AP-1 can be activated by different signaling pathways in different cells. Such results stress the importance of studying transformation in appropriate tissue-specific models because transformation likely occurs via distinct molecular pathways in different cell types.

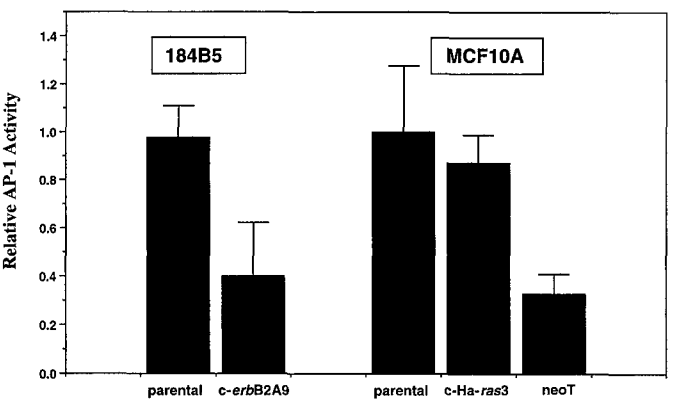

Fig. 8. AP-1 transactivating activity in oncogene-transformed stable clones. Continuously growing cells were cotransfected with −73 ColCAT reporter plasmid and CMV-βGal. CAT activity was measured, and AP-1 activity is shown relative to parental cells. *Bars*, SD.

Some possible explanations for the observed difference in the levels of AP-1 in normal and malignant HMECs include the following:

(*a*) Normal cells secrete autocrine growth factors that increase their basal AP-1 activity. The chronically high AP-1 transactivating activity in normal and immortal HMECs may be due to autocrine stimulation by growth factors as reported by other studies (35, 36). Malignantly transformed HMECs such as MCF10AneoT have lost this autocrine loop (33) and, therefore, are not continuously stimulated by endogenous growth factors. The 184B5 cells, which do not use the TGF-α autocrine loop, may be using other growth factors that activate AP-1. In our study, the use of suramin, a nonspecific growth factor antagonist, effectively inhibited the high AP-1 transactivating activity in immortal HMECs grown without growth factor supplements. This finding suggests that autocrine growth factors, possibly other than TGF-α, are chronically activating signal transduction pathways leading to higher AP-1 activation.

(*b*) Normal and immortal HMECs may be more sensitive than breast cancer cells to decreases in the level of AP-1 expression and activity. Normal and immortal HMECs may require a higher level of AP-1 activity than cancer cells to maintain growth and proliferation. Such a requirement may render nonmalignant HMECs more sensitive to fluctuations of the level of AP-1 activity. The possible requirement of immortal HMECs for high AP-1 activity to maintain their growth is supported by preliminary results from our laboratory that suggest that immortal HMECs are more sensitive to the growth-inhibitory effects of a c-*jun* dominant-negative mutant than are breast cancer cells.

(*c*) Breast cancer cells may use other signal transduction pathways. As normal cells acquire the genetic changes that lead to transformation, they may become less dependent on AP-1-mediated signal transduction pathways for continued growth. This may be reflected in the lower AP-1 expression and activity observed in oncogene-transformed and tumorigenic breast epithelial cells. Nonmalignant HMECs may require the AP-1 pathway for growth and proliferation, whereas breast cancer cells may use other signal transduction pathways to support their growth.

(*d*) High AP-1 activity in breast cancer cells may be inhibitory to the their growth. TPA, which is a potent activator of AP-1, has been shown to inhibit the growth of breast cancer cells (45, 46). In addition, overexpression of c-*jun* and c-*fos* in melanoma and lung cancer cell lines resulted in markedly reduced tumorigenicity and metastatic potential of these cells (47). These data support the hypothesis that the growth of cancer cells may be impaired by persistently high levels of AP-1 activity. Such inhibition of growth may be due to the induction of apoptosis because recent studies have also suggested a critical role of c-Jun in apoptosis in cancer cells (48, 49). Overexpression of a dominant-negative mutant of c-Jun blocked apoptosis induced by stress or ceramide in leukemic cells (48) or by nerve growth factor deprivation in pheochromocytoma cells (49). The mechanism by which c-Jun mediates apoptosis in cancer cells is still unknown, but activated c-Jun may induce the expression of proteins required for the apoptotic response in malignant cells. Because AP-1 complex is formed by dimers of Jun and Fos family members, the expression of c-Jun or c-Fos alone is not a final indication of the level of AP-1 activity. High c-Fos expression in normal and immortal HMECs may be the cause of high AP-1 activity in these cells. However, it is not yet clear that the high AP-1 activity is due to c-Fos, c-Jun, or other Jun/Fos family members. Studies are now ongoing to determine which Jun/Fos proteins are most critical for the high AP-1 activity in normal breast cells. Other proteins that could dimerize with c-Jun or c-Fos, such as cAMP-responsive element binding protein/activating transcription factor members, may also be contributing to the high AP-1 DNA-binding activity present in normal HMECs. In addition, the lower AP-1 activity present in tumor cell lines that have relatively high AP-1 DNA-binding activity (such as MCF7Adria or MDA MB231 cells [see Chen *et al.* (27)] could be due to the presence of inhibitory proteins that dimerize with AP-1. Such inhibitory proteins that can dimerize with Jun or Fos proteins include other Jun and Fos family members [Jun D (18), ΔFosB (17), and Fra1 or Fra2 (42)] as well as other Jun and Fos dimerizing partners such as cMaf (50), or the small Maf proteins that lack the Maf transactivation domain (MafK, MafF, and MafG; Ref. 51).

Although the present report characterizes the differences in expression and activity of AP-1 transcription factor complex in HMECs *in vitro*, it is possible that AP-1 expression and activity in HMECs *in vivo* is different from that seen *in vitro*. For the present experiments, proliferating normal HMECs were used. Because these cells have a finite life span and retain many of the characteristics of normal breast epithelial cells, we consider them to be the best example of normal breast epithelial cells presently available. However, normal, nonproliferating breast cells *in vivo* may not show such high AP-1 activity. To determine the expression and activity of Jun and Fos proteins *in vivo*, it will be necessary to study their expression and functional activity in normal and malignant breast tissue specimens.

The modulation of AP-1 activity that occurs during transformation of HMECs, as reflected by the difference in AP-1 transactivating activity, may also be occurring in other signal transduction pathways present in these cells. By using these HMECs as a model for carcinogenesis, we may be able to understand the signal transduction pathways and transcription factors involved in the transformation process of breast epithelial cells. The characterization of these critical signal transduction pathways may lead to the identification of novel targets for the treatment or chemoprevention of breast cancer.

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Inhibition of AP-1 Transcription Factor Causes Global Signal Transduction Blockade and Inhibits Breast Cancer Growth

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Abbreviations: AP-1, activating protein-1; TAM-67, cJun Dominant-negative mutant; Dox, doxycycline; ER, estrogen receptor; ERE, estrogen response element; EGF, epidermal growth factor; IGF, insulin-like growth factor; TGF-α; transforming growth factor-α; TPA, 12-O-tetradecanoylphorbol-13-acetate; bFGF, Basic fibroblast growth factor; HRG-β, Heregulin-beta;

Abstract

AP-1 transcription factors play a critical role in signal transduction pathways in many cells. We have investigated the role of AP-1 in controlling proliferative signals in breast cells, and have previously shown that AP-1 complexes are activated by peptide and steroid growth factors in both normal and malignant breast cells. In this study, we investigated the role of AP-1 in transducing proliferative signals induced by peptide and steroid growth factors. We used MCF-7 clones that express a specific inhibitor of AP-1, a dominant-negative cJun mutant (TAM67), under the control of an inducible promoter to investigate the role of AP-1 in regulating breast cancer growth. In the presence of doxycyline (Dox), the AP-1 inhibitor was not expressed, and the MCF-7 clones proliferated normally in response to serum stimulation. However, when Dox was withdrawn, TAM67 was expressed, AP-1 activity was inhibited, and serum-induced proliferation was blocked. We next investigated whether the mitogenic response to specific growth factors also requires AP-1. MCF-7 Tet-Off-TAM67 cells were grown in the presence of increasing concentrations of IGF-1, EGF, heregulin-β, bFGF, or estrogen under un-induced and induced conditions. These studies showed that the AP-1 inhibitor completely blocked proliferation in response to the peptide growth factors (IGF-1, EGF, heregulin-β, and bFGF), and partially blocked the response to estrogen. To investigate the effect of AP-1 blockade on *in vivo* tumor growth, we injected the MCF-7 Tet-Off TAM67 cells into nude mice receiving doxycycline to suppress the expression of the AP-1 inhibitor. After the mice developed tumors, they were randomized to either continue to receive Dox or not. In mice not receiving Dox, the expression of TAM67 was induced, and tumor growth was inhibited, while the tumors in mice receiving Dox continued to grow. Analysis of the tumors from these mice showed that the

expression of TAM67 caused reduced proliferation of the breast cancer cells without inducing apoptosis. These results demonstrate that AP-1 blockade supresses mitogenic signals from multiple different peptide growth factors as well as estrogen, and inhibits the growth of MCF-7 breast cancer cells both *in vitro* and *in vivo*. These results suggest that novel agents specifically targeting AP-1 or its activating kinases could be promising agents for the treatment of breast cancer.

Introduction:

Breast cancer is one of the most common malignancies in women, and is the leading cause of death for women between the ages of 40 and 55 in the United States (1,2). During the last two decades, breast cancer has been intensively studied, and recently new treatments for this disease have emerged. Drugs that inhibit the ability of estrogen to activate the estrogen receptor (e.g., tamoxifen) are used to prevent and treat breast cancer. Drugs that block growth factor receptors, such as antibodies specific for the epidermal growth factor receptor, or for ErbB2 (Her2/neu), have previously been shown to inhibit breast cancer cell proliferation (2-4) and are now being used to treat breast cancer patients. These drugs target specific molecules, which are expressed by only a sub-set of breast cancers. Many of these drugs inhibit individual signal transduction pathways, and may ultimately be ineffective, since several different signal transduction pathways can stimulate breast cell proliferation. It may be more effective to inhibit signal transduction at a more distal point in the cascade, where many mitogenic signals converge. Since transcription factors, the nuclear proteins that control DNA transcription and gene expression, are the most distal components of these converging mitogenic signal transduction pathways, they could be good targets for new therapeutic agents.

In this study we investigated whether inhibition of the AP-1 transcription factors suppresses breast cancer growth. The AP-1 family is a key family of transcription factors transducing multiple signals, including mitogenic and stress induced signals. These transcription factors are complexes of DNA-binding proteins made up of homodimers of Jun family members or heterodimers of Jun and Fos family members. AP-1 functions by regulating AP-1-dependent downstream genes, or by interacting with transcriptional co-activators or integrators, such as Jab-1,

CBP or p300 (5,6). AP-1 transcription factors are expressed in most cell types, and are activated by specific kinases, which are themselves activated by diverse signals such as growth factor stimulation, exposure to UV light, oxidative stress, tumor promoters, or oncogene overexpression or activation (7).

In fibroblasts, AP-1 plays a critical role in cell proliferation. The expression of both c-*jun* and c-*fos* is rapidly increased in many cell types in response to mitogens such as serum or EGF (7-9). Microinjection and knockout experiments have shown that both Jun and Fos protein are necessary for fibroblast growth. Bravo and coworkers showed that microinjection of Fos- or Jun family member- specific antibodies blocks DNA synthesis and S phase entry in fibroblasts (10,11). Other studies using c-jun null mutation mouse embryonic fibroblasts demonstrated that these cells have reduced growth and lose the response to growth factor stimulation (12). These results suggest AP-1 complex is necessary for the proliferation of these cells.

In breast cells, previous studies have suggested that growth factors and hormones, such as IGF, EGF, estrogens and retinoids, can modulate AP-1 transcriptional activity (13-16). Other studies demonstrate that ER and AP-1 interact to regulate the expression of certain estrogen- and/or tamoxifen-regulated genes (17). Activation of AP-1 may also contribute to tumor cell invasive capacity (18) and tamoxifen resistance (18-21). These previous studies provide indirect evidence to suggest that the AP-1 transcription factor is an important regulator of breast cancer cell growth, invasion, and resistance to anti-estrogens.

To directly investigate whether AP-1 controls breast cell growth, we have used a specific inhibitor of AP-1, the dominant negative c-Jun mutant, TAM67, to block AP-1 activity in breast cancer cells. We have previously investigated the effect of AP-1 blockade on the growth of different breast cells using TAM67 (22). Results from these studies demonstrated that TAM67 blocks AP-1

activation in normal, immortal and malignant breast cells. In the present study we have explored the role of AP-1 in controlling the *in vitro* and *in vivo* growth of MCF-7 breast cancer cells. For these experiments, we used MCF-7 clones that express TAM67 under the control of an inducible promoter. Using these clones, we demonstrated that the expression of TAM67 inhibited AP-1 activity and inhibited MCF-7 cell growth *in vitro*. AP-1 blockade also completely suppressed MCF-7 cell growth induced by these peptide growth factors, and partially inhibited growth in response to estrogen. Studies of MCF7 xenografts in nude mice demonstrated that AP-1 blockade also inhibited the MCF-7 tumor growth in *vivo*. Investigation of the tumors from these mice showed that TAM67 caused decreased proliferation of the breast cancer cells without inducing apoptosis. Thus, AP-1 blockade inhibits the growth of MCF-7 breast cancer cells both *in vitro* and *in vivo*. These studies also suggest that agents that block AP-1 activation could be promising agents for the prevention or treatment of breast cancer.

Materials and Methods

Cell Culture and Transfection

The generation of the MCF-7 Tet-Off TAM67 Clones #62, #67 and vector clones #1, #3 has been previously described (22). The cells were maintained in Improved MEM (high zinc option, Life Technologies, Grand Island, New York) with 100 μg/ml of geniticin and 100 μg/ml of hygromycin. The cells were transfected using Fugene 6 reagent (Roche, Indianapolis, Indiana) according to manufacturer's recommendations.

Western Blot Analysis

Equal amounts of total cellular protein extract were electrophoresed on a 12% acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane (Bio-Rad, Hercules, California). The primary antibody used was rabbit anti-cJun Ab-1 (Oncogene Science, Cambridge, MA). Donkey anti-rabbit antibody (1:4,000, Amersham, Piscataway, New Jersey) was used as secondary antibody. Blots were developed using the enhanced chemiluminescence (ECL) procedure (Amersham, Piscataway, New Jersey).

Luciferase Assay to Measure AP-1 and ER activity

AP-1 transcriptional activity in cells was measured using the Dual-Luciferase^{TM} Reporter Assay (Promega, Medison, Wisconsin) according to manufacturer's protocol. The cells were co-transfected with the Col-Z-Luc reporter gene containing the luciferase gene linked to 1100 bp of the human collagenase gene promoter which contains a single AP-1 binding site (TGAG/CTCA) and pRL-TK, a Renilla construct for normalizing of transfection efficiency. To determine the AP-1 activity stimulated by growth factors, the cells were treated with EGF (100 ng/ml, Life

Technologies, Grand Island, New York), IGF-1 (100 ng/ml, GroPep, Australia), heregulin-β1 (10 ng/ml, R&D System, Minneapolis, Minnesota), bFGF (10 ng/ml, Life Technologies, Grand Island, New York), 17-β-estradiol (10^{-9}M, Sigma, St. Louis, Missouri), or DMSO, respectively for 6 hours before harvest. Transfected cells were lysed 36 hours after transfection and luciferase activity was measured with equal amounts of cell extract using a microplate luminometer (Labsystems, Helsinki, Finland) and normalized with the Renilla activity.

To measure estrogen receptor activity, the Vit-ERE-TK-Luc construct was employed instead of Col-Z-Luc to perform the luciferase assay. The cells were starved of estrogen for at least 24 hours in phenol red-free medium with 5% charcoal-stripped serum, and then treated with 17-β-estradiol (10^{-9}M) for 12 hours to stimulate the ERE activity before harvest.

Cell Growth Assays

Cell proliferation assay of stably transfected and Tet-Off cell lines

The CellTiter 96^{TM} AQueous Non-Radioactive Cell Proliferation Assay (MTS assay; Promega, Madison, WI), performed according to manufacturer's protocol, was used to measure breast cancer cell growth. Approximately 12,000 cells were seeded in wells a 24 well plate and doxycycline or vehicle was added to block or induce the expression of TAM67 by MCF-7 Tet-Off TAM67 cells. A solution containing a 20:1 ratio of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium) and PMS (phenazine methosulfate) was added to the cells for 2 hours at 37° C and absorption at 495 nm was determined. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Cell proliferation assay of breast cells treated with specific growth factors

The MTS assay described above was used to measure MCF-7 breast cancer cell growth after stimulation with specific growth factors, including EGF (0 to 100 ng/ml), IGF-1 (0 to 100ng/ml), heregulin-β1 (0 to 10 ng/ml), bFGF (0 to 10ng/ml), or estradiol (0 to 10^{-9}M), respectively. The cells were cultured in medium with or without doxycycline for 4 days, and then seeded in 24 well plates in full medium. The cells were allowed to attach overnight, and then starved for 24 hours in 5 % charcoal-stripped or serum-free (with 10 mM Hepes; 1 μg/ml transferrin; 1 μg/ml fibronectin; 200 μM glutamine; 1x trace element Biofluids Division, BSI, Rockville Maryland), phenol red-free medium. The cells were then cultured at 37° C for 0 to 8 days with different concentrations growth factors or estrogen. Cells were harvested every other day and the MTS assay was done as described above to measure proliferation. Each data point was performed in quadruplet, and the results were reported as mean absorption +/- standard error.

Nude Mouse Xenograft Experiments

96 athymic Balb/C nude mice (Harlan Teklad, Madison, Wisconsin) were randomized to four groups. Estrogen pellets (Innovative Research of America, Sarasota, Florida) were injected into all animals to stimulate the development and growth of tumors. The next day, mice from each group were injected into the fat pad with approximately $5x10^6$ cells/mouse of four different MCF-7 clones (MCF-7 Tet-Off TAM67 clones #62, #67 and vector clones #1, #3), and fed with Doxycycline-containing water (200 μg/ml). After tumors developed and reached the size of 100 mm^3, the mice were randomized to receive doxycycline-free or doxycycline-containing water to induce or suppress the expression of TAM67. The tumor sizes were measured twice a week and tumor volumes were estimated according to the formula: (long dimension) x (short dimension)^2/2. Tumor growth rates of different groups were calculated and statistically analyzed as described below in Statistical Analysis.

Immunohistochemical Analysis

Tumor tissues were collected from sacrificed nude mice. The samples were fixed in 10% neutral buffered formaldehyde overnight and then embedded in paraffin. Tissue sections were then mounted on slides and processed for either hematoxylin & eosin staining or immunohistochemical staining. For immunohistochemical studies, tissue sections were cut at 4 μm and mounted onto slides. Slides were deparaffinized, and then endogenous peroxidase was blocked with 0.1% sodium azide in 3% hydrogen peroxide in 1 x auto buffer. Slides were then rinsed in PBS, and nonspecific binding was blocked with 10% albumin. Because the TAM67 gene was FLAG-tagged, an anti-FLAG antibody was employed as first antibody (1:10,000, M2, Sigma, St. Louis, Missouri), followed by a biotinylated rabbit anti-mouse antibody (1:100), and peroxidase activity was visualized using DAB chromagen intensified with 0.2% osmium tetroxide. For immunohistochemical staining of phospho-Histone H3, the anti-phospho-Histone H3 monoclonal antibody (1:400, Upstate, Lake Placid, New York) was employed, followed by biotinylated anti-rabbit antibody (1:200). The slides were counterstaining with 1 % methyl green.

TUNEL Assay

Paraffin-fixed tumor tissues were cut at 3-4 microns and mounted to slides. The slides were baked overnight at 58°C and deparaffinized, and were digested in proteinase K for 15 min at 37°C. 3% hydrogen peroxide was used to block endogenous peroxidase. The slides were incubated with avidin solution, then biotin solution for 15 min respectively, and then incubated with TdT reaction cocktail (TdT (Roche, Indianapolis, Indiana), 1:400; Manganese cation, 1:50; d-UTP-biotin 16, 1:100) for 2hrs at 37°C. Freshly prepared horseradish peroxidase labeled streptavidin (Dako, Carpinteria, California) at a 1:200 was added, and peroxidase activity was visualized using DAB chromagen. Counterstaining was done with 0.05 % methyl green.

Statistical Analysis

Tumor growth *in vivo* was approximately exponential, but varied slightly from animal to animal. To compare the growth rates of tumors in animals treated with Dox or not, we estimated individual growth rates by linear regression of logtransformed tumor volumes on time, and then compared the growth rates by student's t-test. Growth rates were summarized by means and 95% confidence intervals.

Results

Expression of cJun dominant-negative mutant inhibits basal and induced AP-1 activity

TAM67 (Fig. 1a) is a mutated form of c-Jun that can specifically inhibit AP-1 activity in different cell types(23). Using this mutant, we previously isolated MCF-7 clones that express TAM67 under the control of an inducible promoter, the tet-off promoter(22). In the present study, we used these MCF-7-Tet-Off-TAM67 clones to determine the effect of AP-1 blockade on breast cancer growth *in vitro* and *in vivo*. First, the expression of TAM67 protein in independent clones was determined using Western Blotting (Fig. 1b). As can be seen, no TAM67 protein was expressed in the presence of doxycycline, while high levels of TAM67 protein were seen when the cells were cultured in the absence of doxycycline.

To demonstrate the effects of TAM67 on basal AP-1 activity and AP-1 activity induced by peptide growth factors and estrogen, we performed luciferase reporter assays using an AP-1-dependent reporter construct in the presence and absence of doxycycline in our MCF-7 TAM67 Tet-Off cell clones #62 and #67. For these experiments, the cells were starved of all growth factors and stimulated with the individual growth factors (EGF, IGF-1, HRG-β, bFGF or estradiol). These studies demonstrated that all tested growth factors stimulated AP-1 activity in MCF-7 cells (Fig. 1c). TAM67 repressed the basal level of AP-1 activity, and also inhibited AP-1 activity induced by each of these growth factors and estrogen (Fig. 1c).

TAM67 inhibits MCF-7 cell growth induced by serum and by polypeptide growth factors

We next examined whether TAM67 expression inhibited MCF-7 breast cancer cell growth induced by serum or by individual growth factors. First, we tested the effect of TAM67 on

serum-induced MCF-7 cell growth. Different concentrations (0.5, and 5.0 %) of fetal bovine serum were used to stimulate the growth of serum-starved MCF-7 Tet-Off TAM67 clone cells (#62), and vector-transfected cells (clone #1), in the presence or absence of doxycycline. We found the expression of TAM67 after the removal of Dox from the culture medium totally inhibited MCF-7 cell growth stimulated by fetal bovine serum. These cells proliferated normally after serum stimulation in the presence of Dox (#62 +Dox). Vector transfected cells grew well after serum stimuation in the presence or absence of Dox (Fig. 1d).

To determine whether AP-1 blockade inhibited proliferation induced by specific peptide growth factors, we treated the MCF-7 cells with different concentrations of peptide growth factors to stimulate cell growth in the presence or absence of Dox (EGF, 0-100 ng/ml; IGF-1, 0-100 ng/ml; heregulin-β, 0-10 ng/ml; bFGF, 0-10 ng/ml). The results from these experiments showed that all these peptide growth factors stimulated the proliferation of both vector- and TAM67-transfected MCF-7 cells (Fig. 2). When the MCF-7 Tet-Off TAM67 cells were cultured in the absence of Dox, TAM67 was induced and the cell growth stimulated by growth factors EGF, IGF-1, heregulin-β, bFGF was totally inhibited (Fig. 2a, 2b, 2c and data not shown for bFGF). The vector-transfected cells responded equally well to these growth factors in the presence or absence of Dox (Fig. 2).

TAM67 partially inhibited estrogen-induced MCF-7 cell growth

MCF-7 breast cancer cells express the estrogen receptor and proliferate in response to estrogen stimulation. This proliferative response is caused by estrogen activating the estrogen receptor, which may or may not involve the AP-1 transcription factor. AP-1 and the estrogen receptor have been previously shown to affect each other through transcription factor cross talk (13, 17, 24, 25. To investigate the effect of AP-1 blockade on estrogen-induced gene expression, we

used the Vit-ERE-TK-Luc reporter construct which has a classical ERE upstream of the TK promoter and luciferase gene to measure estrogen-induced gene expression. The TAM67 clones #62, #67 and Vector clones #1, #3 were transfected with this estrogen-inducible construct, and were treated or not treated with estrogen in the presence and absence of Dox. All these clones responded well to estradiol, both in the presence and absence of Dox (Fig. 3a). The results demonstrated that AP-1 blockade induced by expression of TAM67 did not block estrogen receptor transactivating activity. Thus, the inhibition of estrogen-induced growth by TAM67 is likely due to blocking signals independent or downstream of classical estrogen-induced transcription.

To investigate whether AP-1 blockade inhibits estrogen-induced growth, we measured the proliferative response to estrogen using our MCF-7 Tet-Off TAM67 cell line. MCF-7 Tet-Off TAM67 cells and vector-transfected cells were treated with different concentrations of estradiol (0-10^{-9}M) to stimulate cell growth in the presence or absence of Dox. The results from these experiments showed that estrogen stimulated the proliferation of MCF-7 cells and that the growth was dose-dependent both in the vector clone and the TAM67-expressing clone (Fig. 3b). When the MCF-7 Tet-Off TAM67 cells were cultured in the absence of Dox, TAM67 was induced and the cell growth stimulated by estrogen was suppressed. However, while TAM67 totally inhibited peptide growth factor-induced growth, it did not totally block estrogen-induced growth. (Fig. 3b).

TAM67 inhibits MCF-7 xenograft tumor growth in nude mice

Since TAM67-induced AP-1 blockade inhibited MCF-7 cell growth, we next investigated whether TAM67 could also inhibit breast tumor growth *in vivo* in nude mice. For these experiments, we utilized two MCF-7 Tet-Off TAM67 cell clones (clones #62, #67) and two vector clones (#1, #3). These cells were injected into the mammary fat pad of nude mice that received estrogen pellets to stimulate the development and growth of tumors as described in Materials and Methods. After the

tumors developed in nude mice and the tumor sizes were greater than 100 mm^3, we randomized the mice of each group to either receive or not receive doxycycline to suppress or induce the expression of TAM67. Tumor size was then measured. The size of the tumors as a function of time is shown in Figs. 4a and 4b. As can be seen, tumors from vector-transfected cells grew rapidly when the mice were fed with water containing or not containing Doxycycline (Fig. 4a). In the presence of Dox, the TAM67 tumors also grew well. However, when the mice were fed with water without Dox, the tumor growth was dramatically reduced (Fig. 4b).

We also calculated and compared the growth rates of tumors in each group. There was no significant difference in the average growth rate of the vector-transfected clones treated with or without Dox (Fig. 4c). However, in both TAM67-transfected clones, tumor growth rates were significantly lower in the absence of Dox. These results demonstrate that AP-1 blockade in established breast tumors suppresses their growth *in vivo*.

We next examined the histologic appearance of these tumors. No obvious necrosis was observed when TAM67 was induced (Fig. 4d). We can see strong expression of TAM67 (as seen by immunohistochemical staining for the FLAG tag) in tumor tissues collected from TAM67-mice that were fed with water without Dox, while in tumor tissues from TAM67 mice that were fed with doxycycline-containing water and from Vector-mice, there was no expression of TAM67.

TAM67 inhibits proliferation without inducing apoptosis

To better understand the mechanism by which AP-1 blockade affects the growth of breast cancer cells, we investigated whether TAM67 inhibited proliferation or induced apoptosis in the mouse tumor tissues. Phosphorylation of histone H3 correlates closely with mitosis (26, 27). Thus, we chose immunohistochemical staining using anti-phospho-Histone H3 to determine proliferation

in the tumor tissues. There were fewer cells expressing phospho-Histone-H3 in tumors expressing TAM67, compared to tissures not expressing TAM67, both in TAM67 clones #62 and #67 (Fig. 5a). These differences were statistically significant (Fig. 5b).

We next used the TUNEL assay to measure apoptosis in the tumor tissues. Tumors isolated from mice injected with vector clones and TAM67 clones, and grown in the presence or absence of doxycycline, were studied. We observed no differences of apoptotic rates in any of these tumor tissues (Fig. 5c).

Discussion

The above results demonstrate that expression of a cJun dominant negative protein inhibits peptide growth factor-induced activation of the AP-1 transcription factor, and inhibits breast cancer cell growth. In addition, the data show that this AP-1 inhibitor also suppresses estrogen-induced growth of breast cancer cells. AP-1 blockade suppressed the growth of breast cancer cells both *in vitro* and *in vivo* in nude mice. The present results show that this suppression of tumor growth was caused by inhibition of proliferation without inducing apoptosis. These results demonstrate that mitogenic signal transduction in breast cancer cells can be blocked at a distal point at which signals from multiple peptide growth factors and estrogen converge. By blocking signal transduction at the point where these multiple signals converge, one can potentially overcome the problems of receptor downregulation or utilization of alternative growth factor pathways that can occur with other agents that target individual growth factor pathways.

Previous studies have demonstrated that the AP-1 transcription factor is an important regulator of proliferation, transformation, and apoptosis, depending on the cell type. We and others have used the cJun dominant negative mutant, TAM67, to investigate the role of AP-1 in several different cell types. These studies have shown that in fibroblasts, AP-1 is an important regulator of proliferation and transformation (28, 29). Other studies, done in neuronal cells and in hematopoeitic cells, show that AP-1 regulates apoptosis (30, 31). The current study demonstrates that in breast cancer cells, AP-1 is a critical regulator of proliferation.

We and others have investigated the function of AP-1 in normal and malignant breast cells. These previous studies have shown that AP-1 family members are expressed in normal and malignant breast cells, that peptide growth factors and estrogen induce AP-1-dependent

transcriptional activation, and that the anti-estrogen, tamoxifen, can also activate the AP-1 transcription factor. Increased AP-1 activity in breast cancer cells can also lead to tamoxifen resistance. Thus, overexpression of cJun induces tamoxifen resistance in MCF7 breast cancer cells (18). In addition, selection for tamoxifen resistance leads to upregulation of AP-1 activity in breast cancer cells. Dumont and coworkers isolated a hormone-resistant clone of MCF7 cells, that were found to be tamoxifen-resistant and have increased AP-1 activity (32). We have also shown that MCF7 xenografts that acquire tamoxifen resistance by being chronically treated with tamoxifen in vivo, develop increased AP-1 activity at the time they develop tamoxifen resistance by increasing the expression and activity of the cJun activating kinase, JNK (20). Studies of the expression and activity of AP-1 in human breast tumors also demonstrate that AP-1 activity is increased in tamoxifen-resistant breast cancer cells. Johnson and co-workers showed that AP-1 DNA binding activity and JNK activity were increased in tamoxifen-resistant human breast cancers as compared to untreated ER-positive breast cancers (19). All of these results show that the AP-1 transcription factor is an important transducer of mitogenic signals in breast cells.

The results reported here represent the first direct demonstration that the AP-1 transcription factor is essential for mitogenic signal transduction induced by many different growth factors (EGF, TGFα, heregulin, bFGF, IGF-1, and estrogen). A possible mechanism for this general block of proliferative signals is shown in Figure 6. As shown in this figure, peptide growth factors bind their respective membrane bound receptors, and activate cytoplasmic signal transduction cascades. These signals are transduced to the nucleus where AP-1 is activated by phosphorylation, and AP-1-dependent genes are induced. TAM-67 is able to block AP-1 activity, block the expression of these AP-1-dependent genes, and ultimately block proliferation induced by these peptide growth factors. In breast cancer cells treated with estrogen, estrogen is able to bind to the

estrogen receptor, and activate estrogen receptor-dependent genes, either through the "classical pathway" of ER-regulated genes, or through a "non-classical pathway" that activates genes that do not have classical EREs within their promoters. The expression of some of these genes, particularly those with an ERE within their promoter (the classical pathway), may not be directly affected by the expression of TAM-67. However, it is possible that these estrogen-induced genes include peptide growth factors or their receptors. In that case, TAM-67 could inhibit the estrogen-induced signal transduction indirectly by inhibiting the subsequent peptide growth factor signals.

Another way TAM-67 could inhibit estrogen-induced proliferation is by blocking the expression of estrogen-induced genes that use the "non-classical pathway" of estrogen regulated genes (see Figure 6). Some genes activated by estrogen do not have classical estrogen response elements, but instead have AP-1 sites, within their promoters (13, 25, 33, 34). Expression of these genes is induced by estrogen binding to the estrogen receptor, which then binds to and activates AP-1 transcription factors. These activated AP-1 complexes bind to the AP-1 sites and induce the expression of these "estrogen-induced" genes. We predict that TAM-67 would inhibit the expression of such estrogen-induced, AP-1-dependent genes. Thus, TAM-67 may inhibit estrogen-induced growth by inhibiting the expression of a subset of estrogen-induced, AP-1-dependent genes that are involved in regulating proliferation.

Given the potent ability of TAM-67 to block peptide hormone-induced breast cell growth, it may be possible to combine agents that block AP-1 with anti-estrogens to obtain total signal transduction blockade. In that case, peptide hormone mitogenic pathways, non-classical ER pathways, and classical ER pathways would all be blocked. Such total blockade may be the most effective way to suppress breast cancer growth and avoid the outgrowth of resistant breast cancer clones.

The AP-1 inhibitor described in these studies would be difficult to develop as a therapeutic agent for the treatment of breast cancer. It might have significant toxicity, and it would need to be delivered to breast cancer cells via gene therapy techniques. A more practical application of the present results would be to use small molecule inhibitors of the upstream activating kinases to block AP-1 activation. Such kinases would include either Jun-N-terminal kinases or MAP kinases. Small molecule inhibitors of these kinases are now being developed and are currently being testing in Phase I trials. Our results suggest that such agents either alone, or in combination with anti-estrogens, have significant promise for the treatment and prevention of breast cancer.

Acknowledgements

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Figure Legends:

Fig. 1a: Schematic diagram of the c-Jun and TAM67 proteins.

Fig. 1b: Expression of TAM67 in MCF-7 Tet-Off TAM67 clones #62 and #67 measured by Western blot as described in "Materials and Methods". The cells were cultured in the medium without Dox for 0-7 days, and lysates prepared and loaded anto the gel. A Western blot to actin was used to demonstrate equal loading.

Fig. 1c: TAM67 inhibition of basal, peptide growth factor-induced, and estrogen-induced AP-1 activity in MCF-7 Tet-Off TAM67 clone #62. The cells were cultured in the presence or absence of Dox for 5 days to suppress or induce the expression of TAM67. The cells were then starved of all growth factors for 2 more days (in the presence or absence of Dox). The cells were then transfected with and AP-1 dependent reporter, and then stimulated with different growth factors for 6 hours. Cell lysates were prepared, and luciferase assays were performed as described in "Materials and Methods".

Fig. 1d: TAM67 inhibition of serum-induced cell growth. MCF-7 Tet-Off TAM67 cells (clone #62) were cultured in the presence or absence of Dox for 5 days, after which time the cells were starved of fetal bovine serum for 2 more days. The cells were then stimulated with serum (at 0.5 % and 5 % concentrations) and cell growth was determined as described in "Materials and Methods". MCF-7 Tet-Off Vector clone #1 was used as control. *indicates statistical significant difference ($P < 0.05$).

Fig. 2. TAM67 inhibition of peptide growth factor-induced cell growth. MCF-7 Tet-Off TAM67 cells (clone #62) or MCF Tet-Off vector (clone #1) were cultured in the presence or absence of Dox

for 5 days, after which time the cells were starved of all growth factors for 2 more days (in the presence absence of Dox). The cells were then stimulated with different concentrations of growth factors. Growth was determined as described in "Materials and Methods". **Fig. 2a:** MCF-7 Tet-Off vector cells stimulated with EGF; **Fig. 2b:** Cells stimulated with IGF-1; **Fig. 2c:** Cells Stimulated with HRG-β.

Fig. 3. TAM67 partially inhibits estrogen-induced cell growth but does not block ER-induced transcriptional activity. **Fig. 3a:** Effects on ERE-activity. MCF-7 Tet-Off TAM67 clones #62, #67 and Vector clones #1, #3 were cultured in the presence or absence of Dox for 5 days, and were starved in phenol red-free medium with charcoal stripped serum for 2 more days. The cells were then transfected with an ERE-luciferase reporter, then were stimulated with 17-β-estradiol overnight. The luciferase reporter assays were performed according to the "Materials and Methods".

Fig. 3b: Effect on estrogen-induced growth. MCF-7 Tet-Off TAM67 cells (clone #62) or MCF-7 Tet-Off vector cells (clone #1) were cultured in the presence or absence of Dox for 5 days, after which time the cells were starved of all growth factors for 2 more days (in the presence or absence of Dox). The cells were then stimulated with different concentrations of 17-β-estradiol, and cell growth was determined as described in "Materials and Methods". MCF-7 Tet-Off Vector clone #1 was used as control.

Fig. 4: TAM67 inhibits MCF-7 xenograft tumor growth in nude mice. The MCF-7 Tet-Off TAM67 clones #62, #67 and vector clones #1, #3 were injected into Balb/C nude mice and maintained as described in "Materials and Methods". When the tumors reached 100 mm^3, the mice were

randomized to either continue to receive Dox or not. Tumor growth was measured and is plotted as a function of time after randomization. Each line indicates the growth of each individual tumor.

Fig. 4a: Growth of MCF-7 Tet-Off vector cells in the presence or absence of doxycycline.

Fig. 4b: Growth of MCF-7 Tet-Off Tam67 cells in the presence or absence of doxycycline.

Fig. 4c: Average growth rates of the MCF-7 tumors in the presence (+) or absence (-) of doxycycline. P values to determine statistical significance are shown.

Fig. 4d: H&E staining and immunohistochemical staining for the Flag tag are shown to demonstrate the expression of TAM67.

Fig. 5: Immunohistochemical staining of phospho-Histone H3 and TUNEL assay in tumor tissues. **Fig. 5a:** Tumor tissues from vector clones or TAM67 clones in the presence or absence of doxycycline were stained with anti-phospho-Histone H3 antibody as described in "Materials and Methods". (1) Vector clone (+) Dox; (2) Vector clone (-) Dox; (3) TAM67 clone (+) Dox; (4) TAM67 clone (-) Dox. **Fig. 5b:** The average percentage of phospho-Histone H3 positive cells in each clone is shown. *indicates statistical significant difference ($P < 0.05$). **Fig. 5c:** The percentage of TUNEL positive (apoptotic) cells of 2 Vector Clones and 2 TAM67 Clones in the presence or absence of Dox is shown. There is no difference in % apoptotic cells between tumors grown in the presence or absence of doxycycline.

Fig. 6. Proposed mechanism by which TAM67 blocks signal transduction In breast cancer cells, peptide growth factor pathways are activated by peptide growth factors binding to their receptors, which activate signal transduction kinases, such as ERK, JNK, p38 and PI3-K. These kinases in turn phosphorylate Jun and Fos proteins, causing activation of AP-1 and induction of genes regulating

proliferation. This pathway is blocked by TAM67 (left). For estrogen-induced growth, there appear to be at least two pathways. In the classical estrogen-induced pathway, estrogen binds to estrogen receptor, which then binds to estrogen responsive elements (ERE) in the promoter of target genes to cause cell growth. This pathway may not be blocked by TAM67 (middle). In the non-classical estrogen-induced pathway (right), estrogen binds to ER, ER interacts with AP-1 and induces the expression of AP-1-dependent genes that have AP-1 sites within their promoters, which promote cell growth. This pathway is likely blocked by TAM67.

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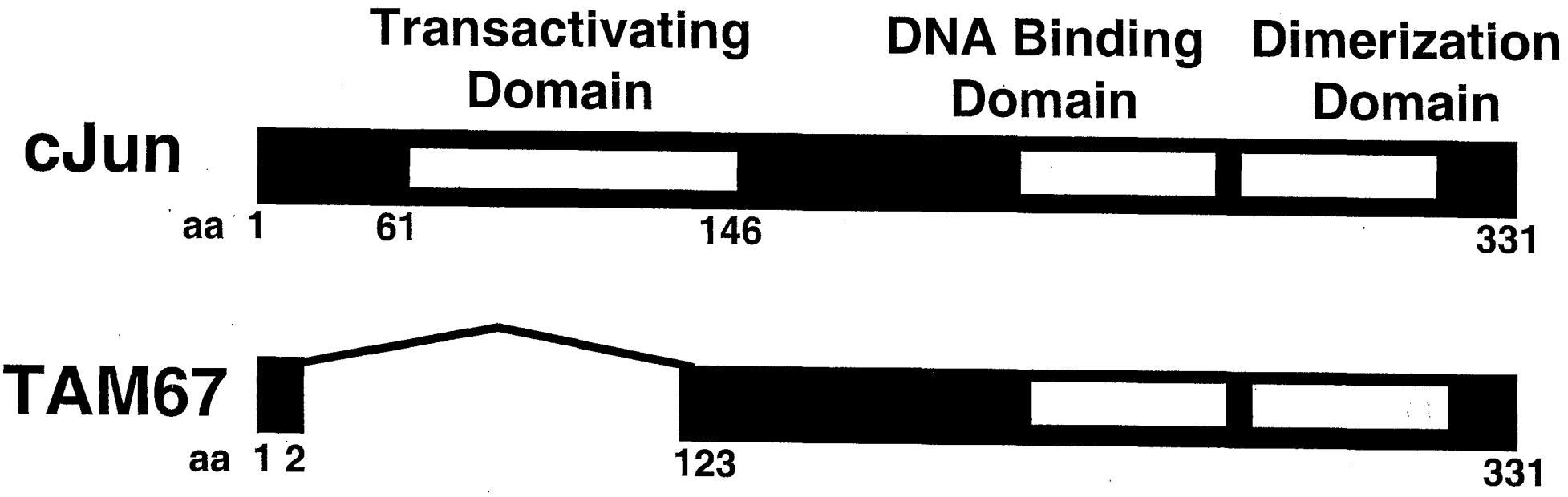
Figure 1A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DNA Binding Domain
Dimerization Domain
cJun
aa 1
61
146
331
TAM67
aa 1 2
123
331

Figure 1B

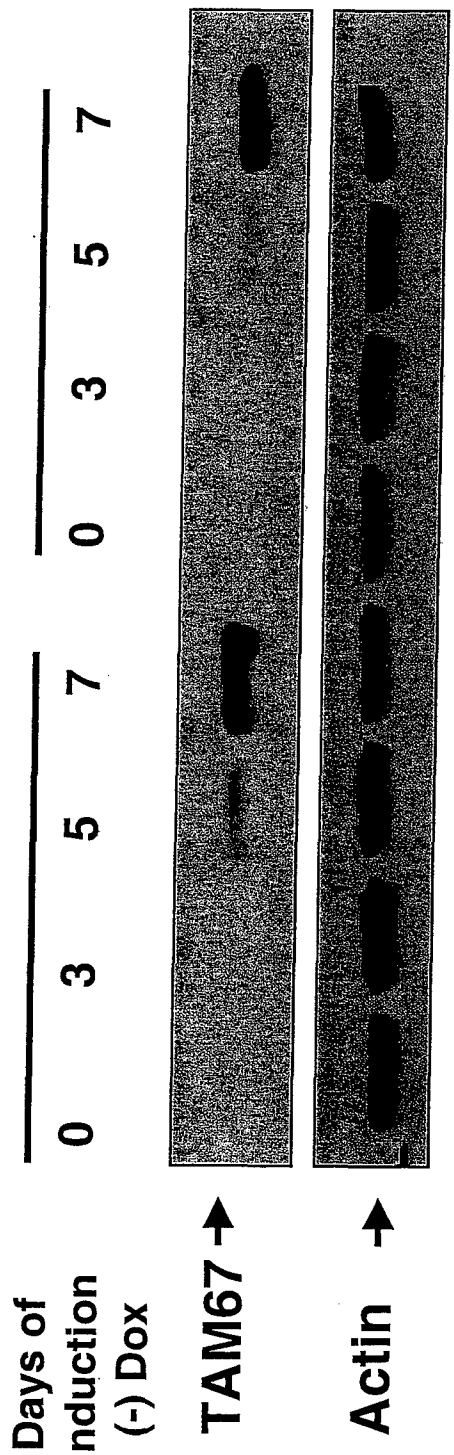
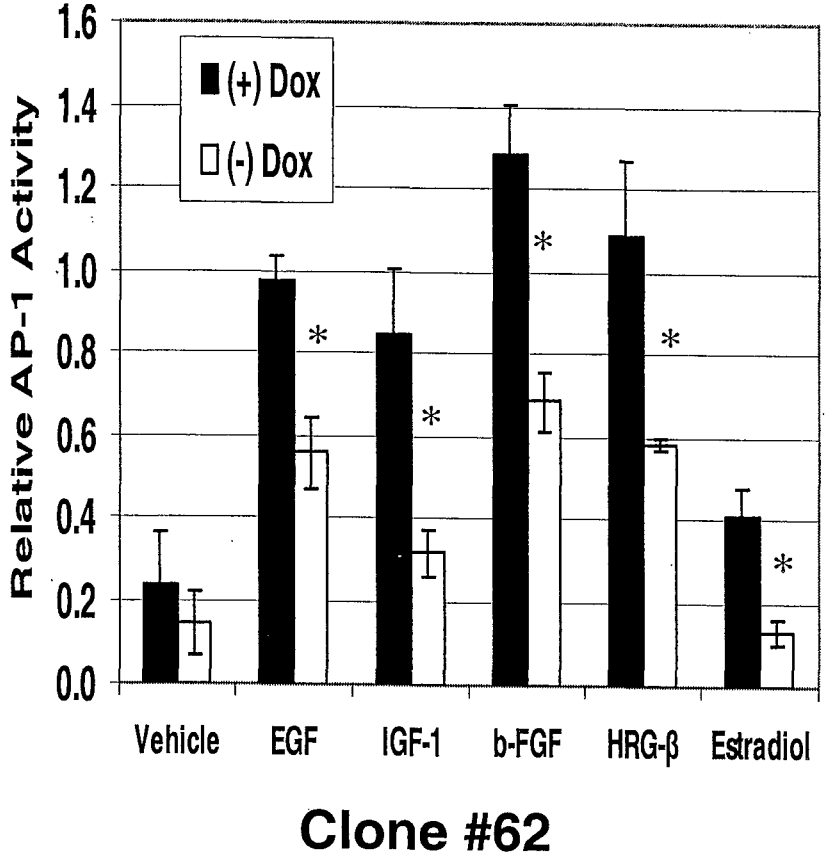

Clone #62
Clone #67
0
3
5
7
0
3
5
7
Days of Induction (-) Dox
TAM67
Actin

Figure 1C



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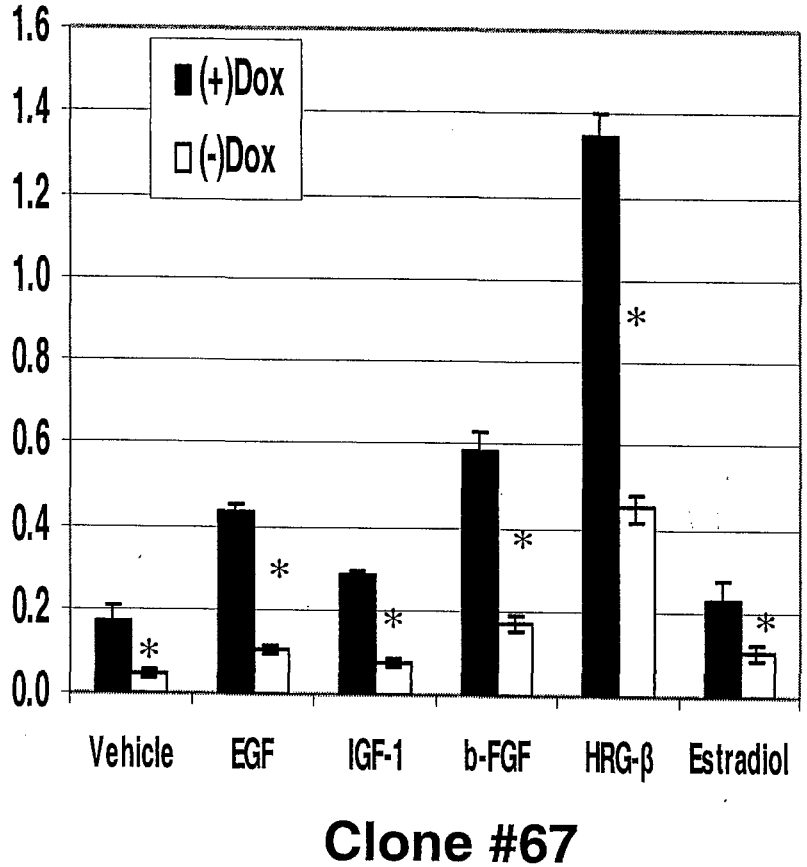

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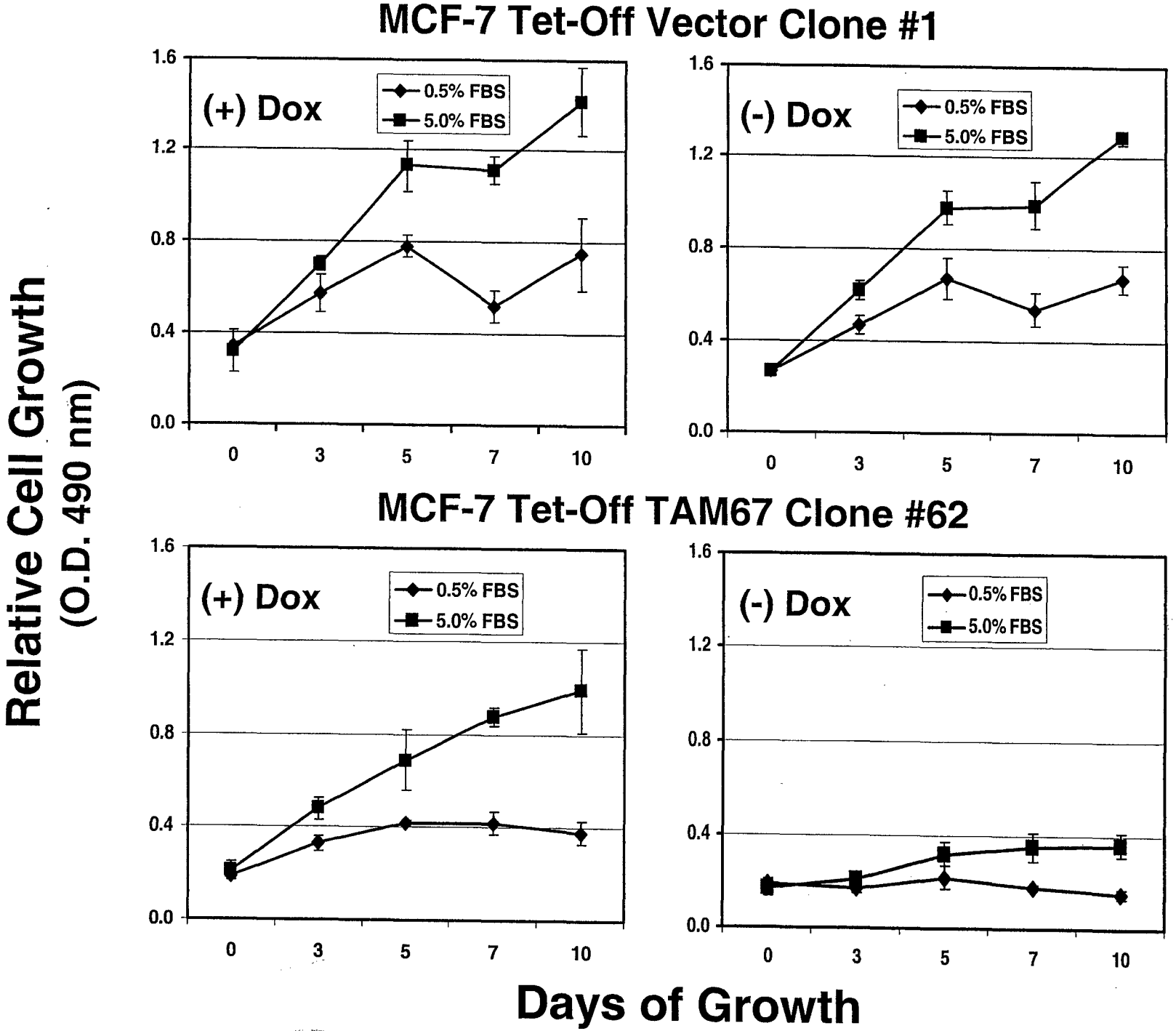
Figure 1D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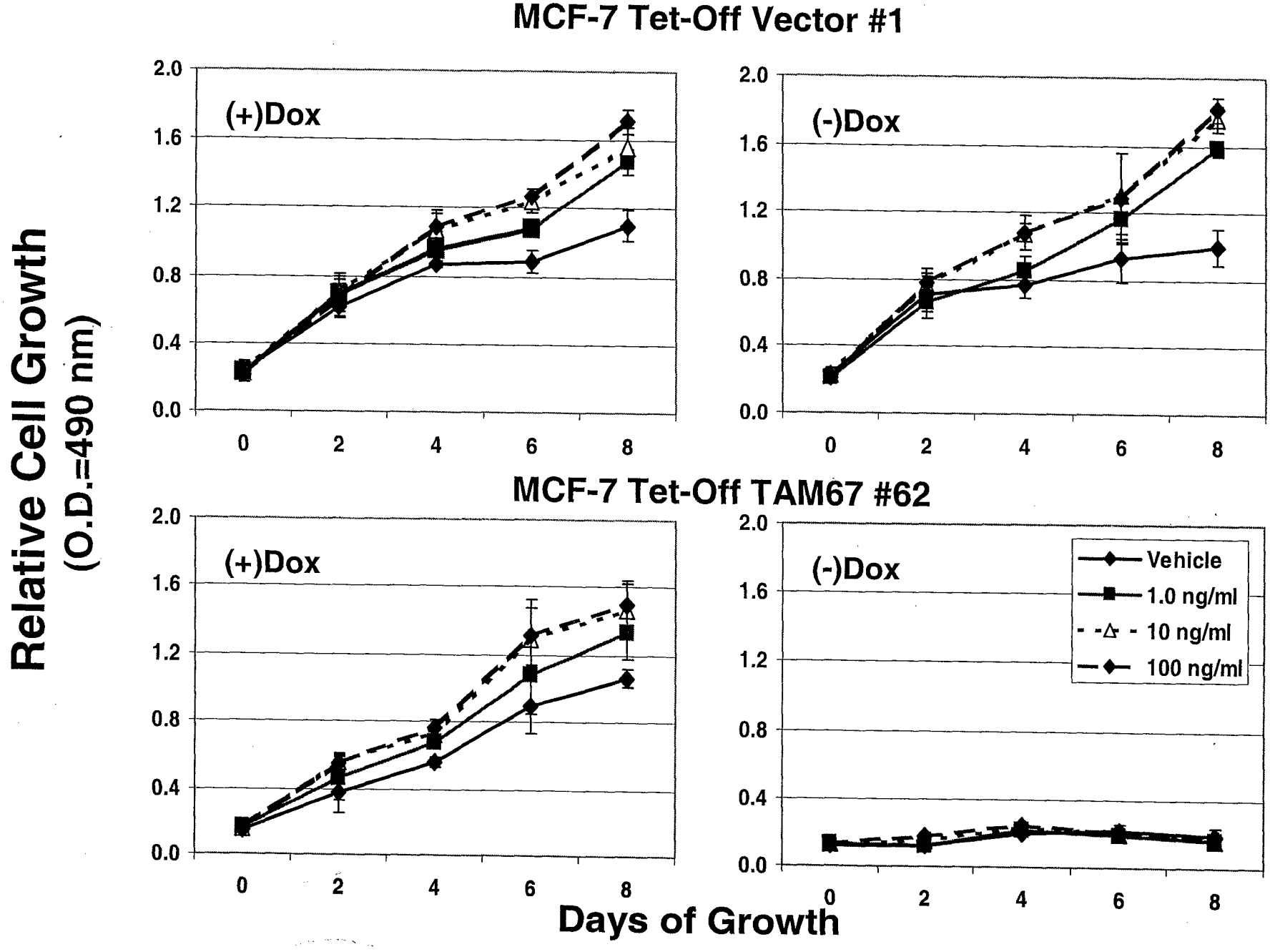
(+) Dox
(-) Dox
0.5% FBS
5.0% FBS
MCF-7 Tet-Off TAM67 Clone #62
Relative Cell Growth
(O.D. 490 nm)
Days of Growth
0.0
0.4
0.8
1.2
1.6
0
3
5
7
10

Figure 2A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(+)Dox
(-)Dox
MCF-7 Tet-Off TAM67 #62
(+)Dox
(-)Dox
Vehicle
1.0 ng/ml
10 ng/ml
100 ng/ml
Relative Cell Growth
(O.D.=490 nm)
Days of Growth
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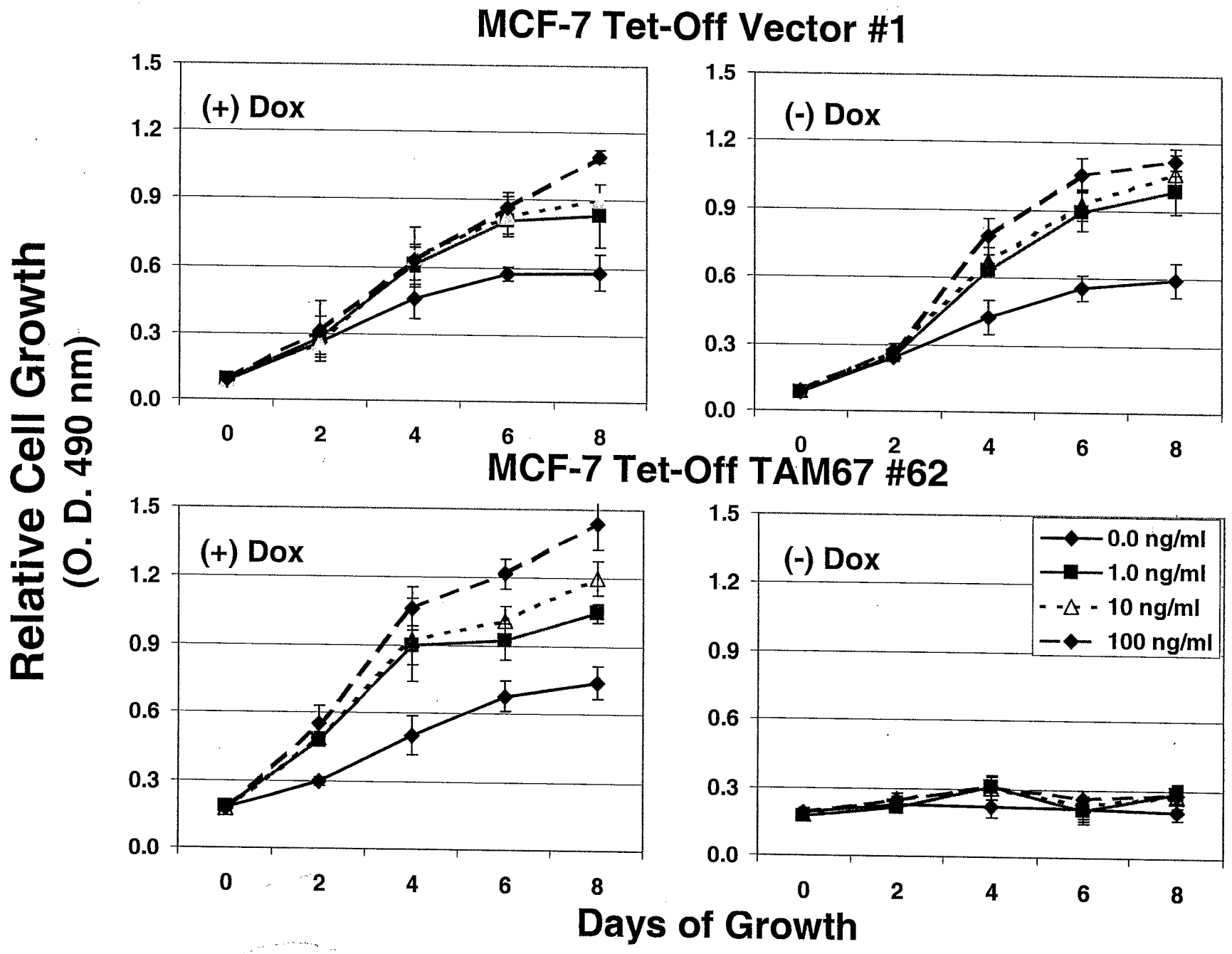
0.4
0.8
1.2
1.6
2.0
0
2
4
6
8

Figure 2B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(+) Dox
(-) Dox
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(+) Dox
(-) Dox
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1.0 ng/ml
10 ng/ml
100 ng/ml
Relative Cell Growth
(O. D. 490 nm)
Days of Growth
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0.6
0.9
1.2
1.5
0
2
4
6
8

Figure 2C

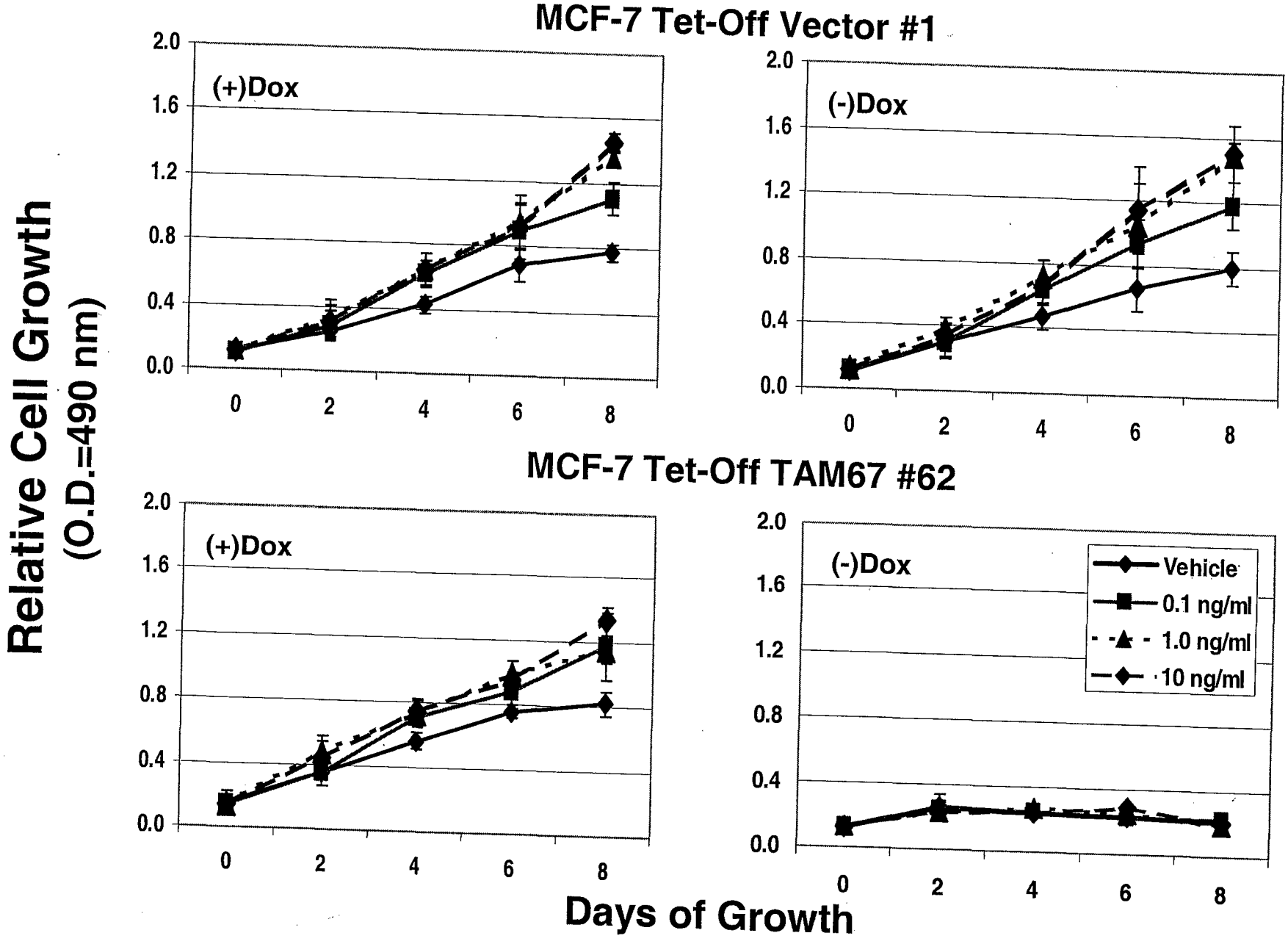
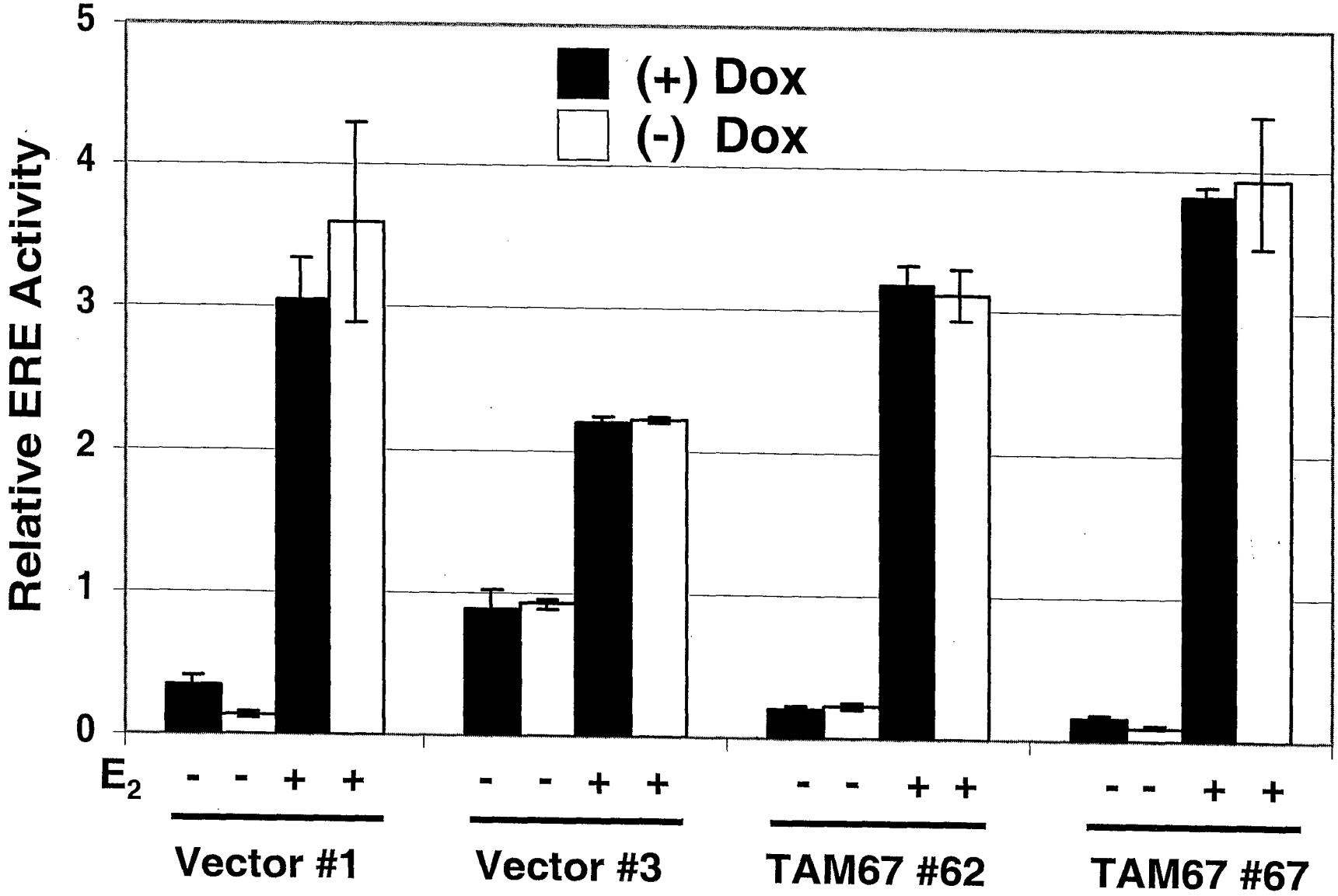

MCF-7 Tet-Off Vector #1
(+)Dox
(-)Dox
MCF-7 Tet-Off TAM67 #62
(+)Dox
(-)Dox
Vehicle
0.1 ng/ml
1.0 ng/ml
10 ng/ml
Relative Cell Growth
(O.D.=490 nm)
Days of Growth
2.0
1.6
1.2
0.8
0.4
0.0
0
2
4
6
8

Figure 3A


Relative ERE Activity
5
4
3
2
1
0
(+) Dox
(-) Dox
E2
- - + +
- - + +
- - + +
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Vector #1
Vector #3
TAM67 #62
TAM67 #67

Figure 3B

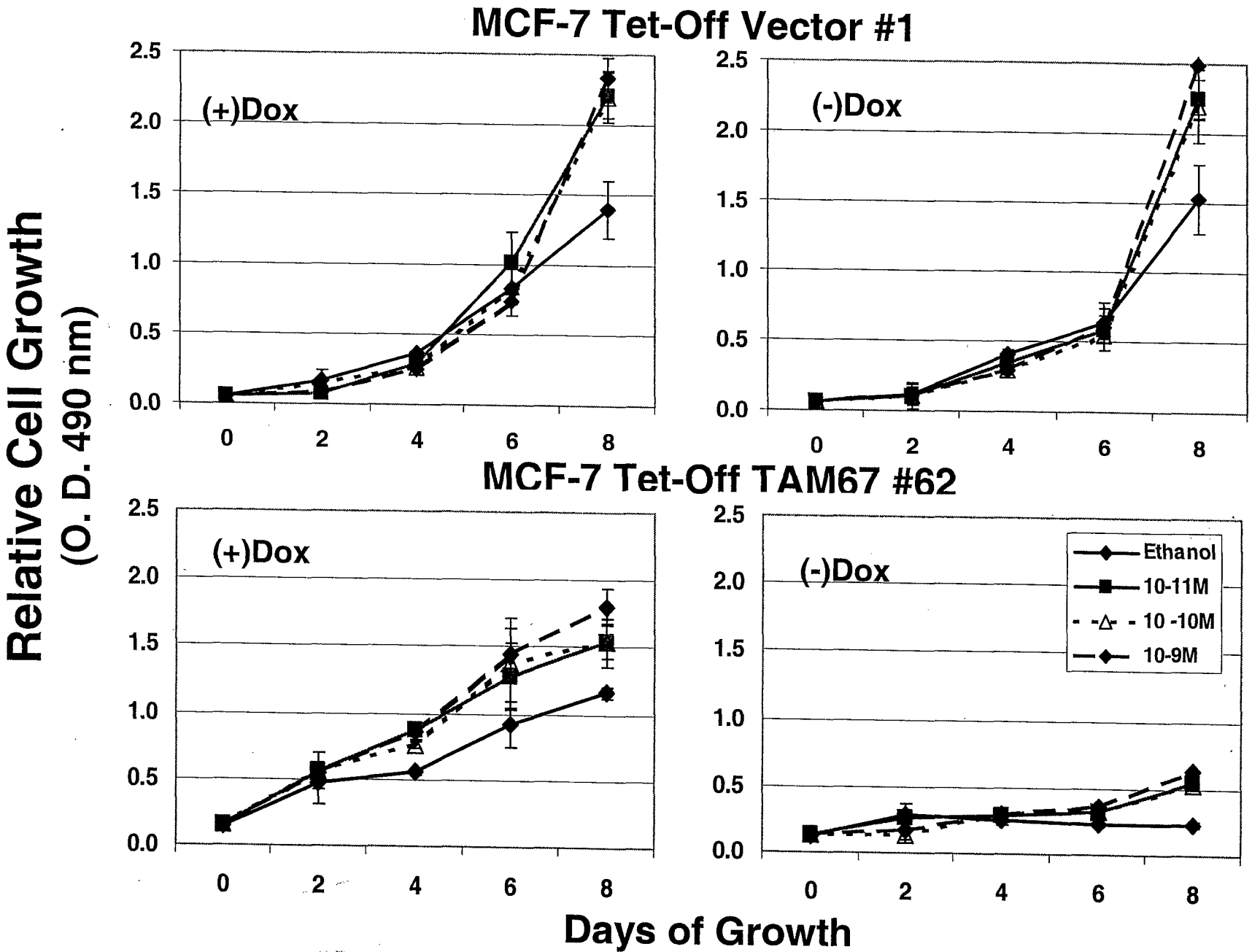

MCF-7 Tet-Off Vector #1
(+)Dox
(-)Dox
MCF-7 Tet-Off TAM67 #62
(+)Dox
(-)Dox
Ethanol
10-11M
10 -10M
10-9M
Relative Cell Growth
(O. D. 490 nm)
Days of Growth
0.0
0.5
1.0
1.5
2.0
2.5
0
2
4
6
8

Figure 4A

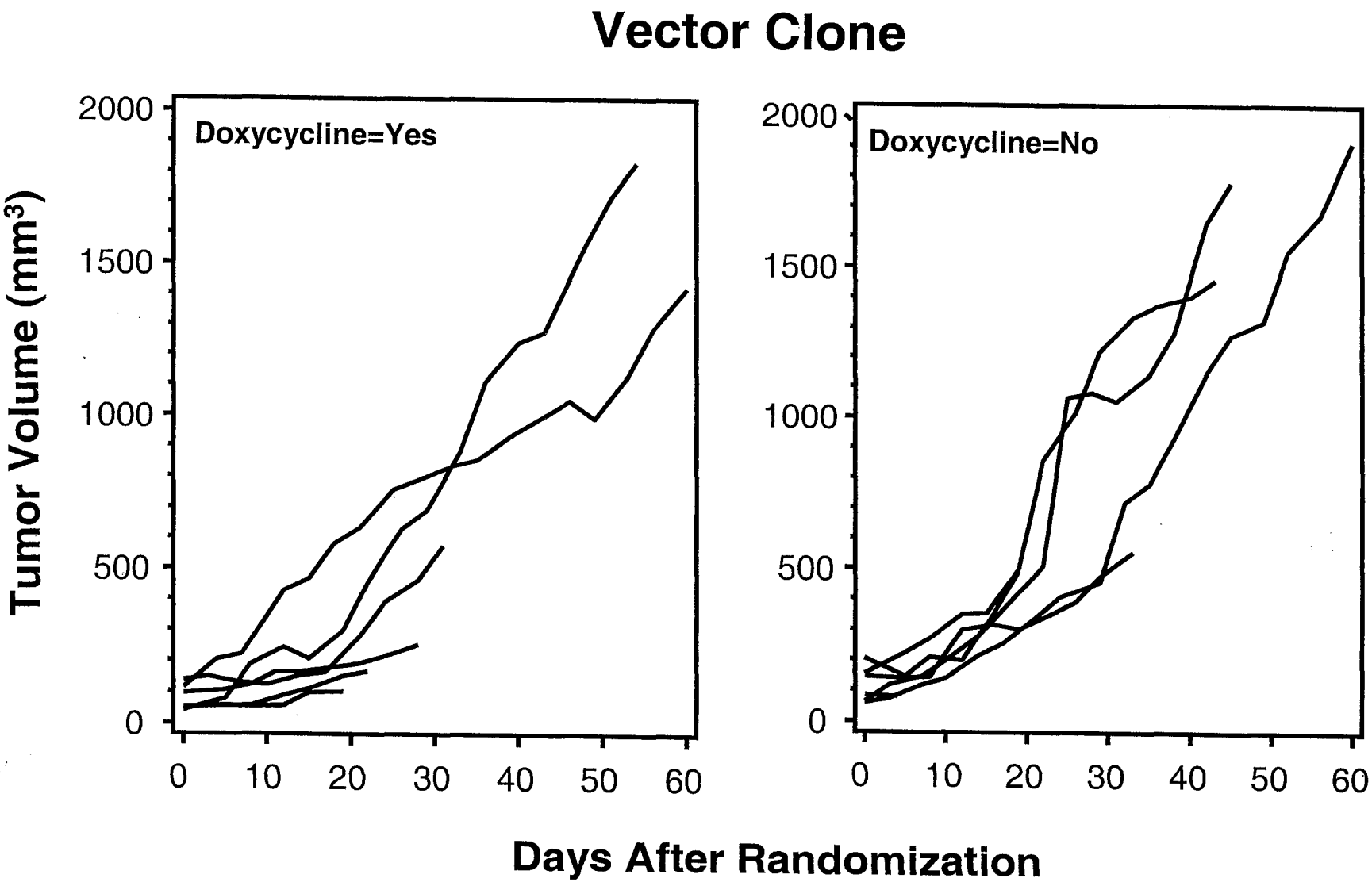

Vector Clone
Doxycycline=Yes
Doxycycline=No
Tumor Volume (mm3)
2000
1500
1000
500
0
0
10
20
30
40
50
60
Days After Randomization

Figure 4B

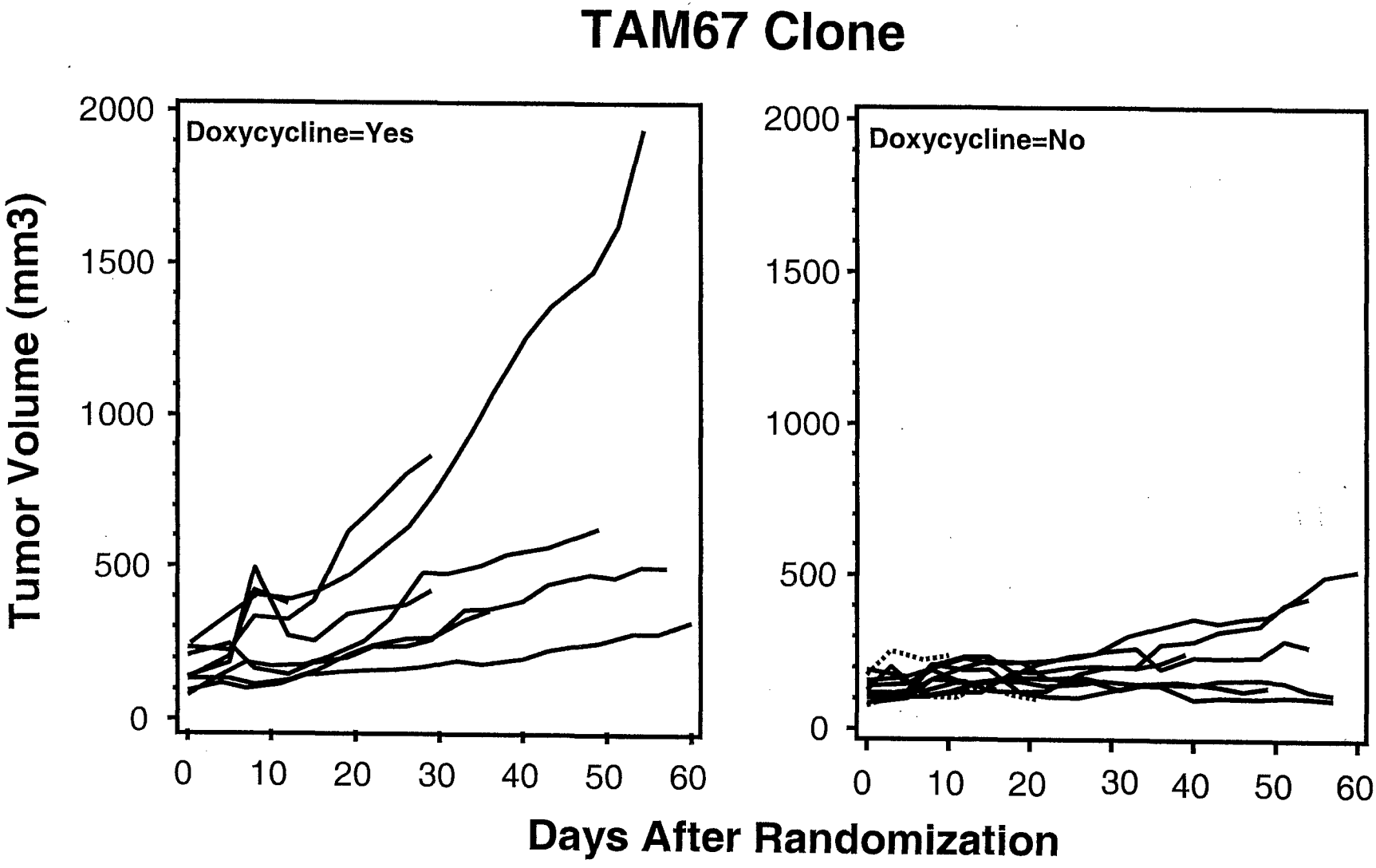

TAM67 Clone
Doxycycline=Yes
Doxycycline=No
Tumor Volume (mm3)
2000
1500
1000
500
0
0
10
20
30
40
50
60
Days After Randomization

Figure 4C

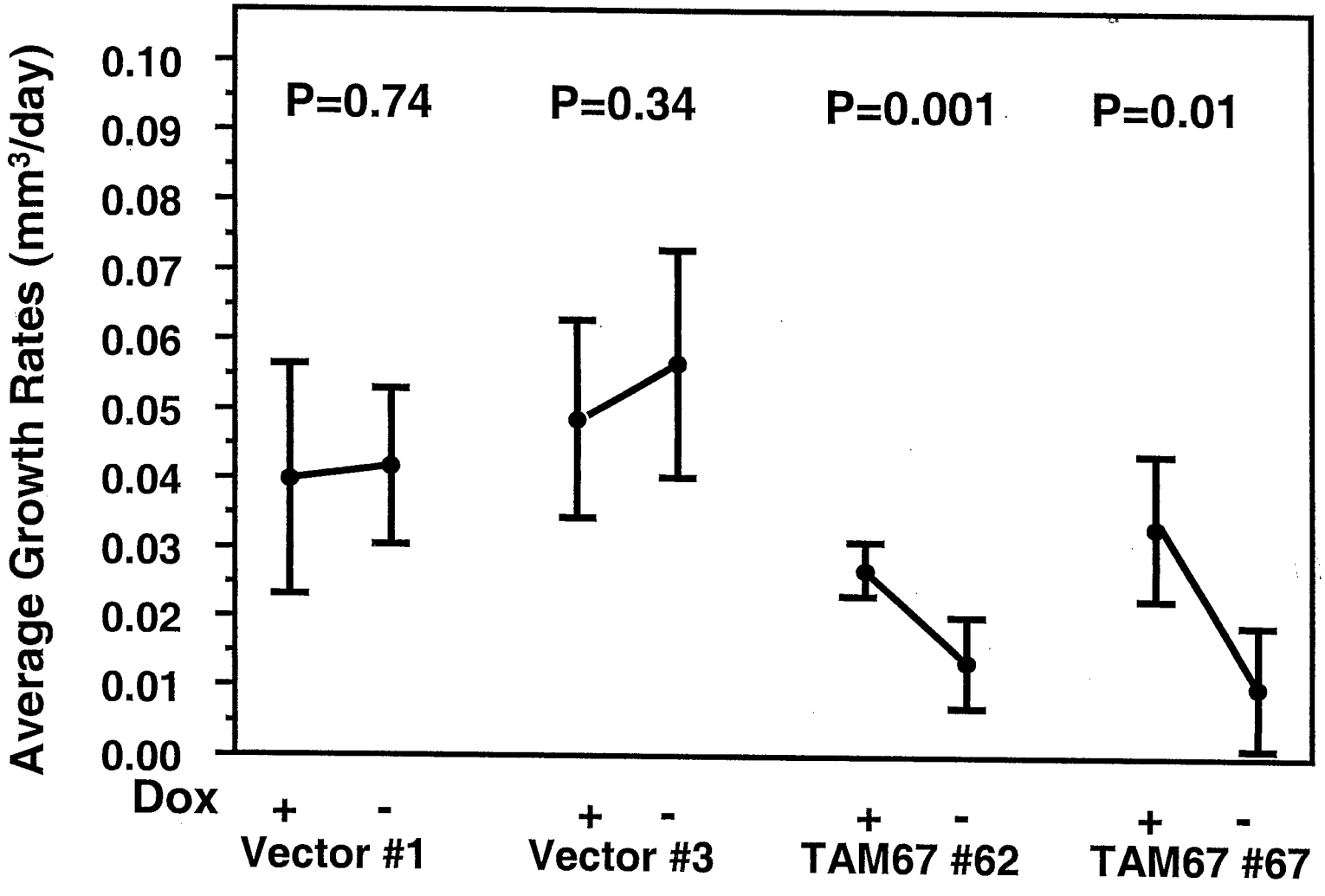

Average Growth Rates (mm³/day)
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
P=0.74
P=0.34
P=0.001
P=0.01
Dox
+
-
Vector #1
Vector #3
TAM67 #62
TAM67 #67

Figure 4D

Figure 5A

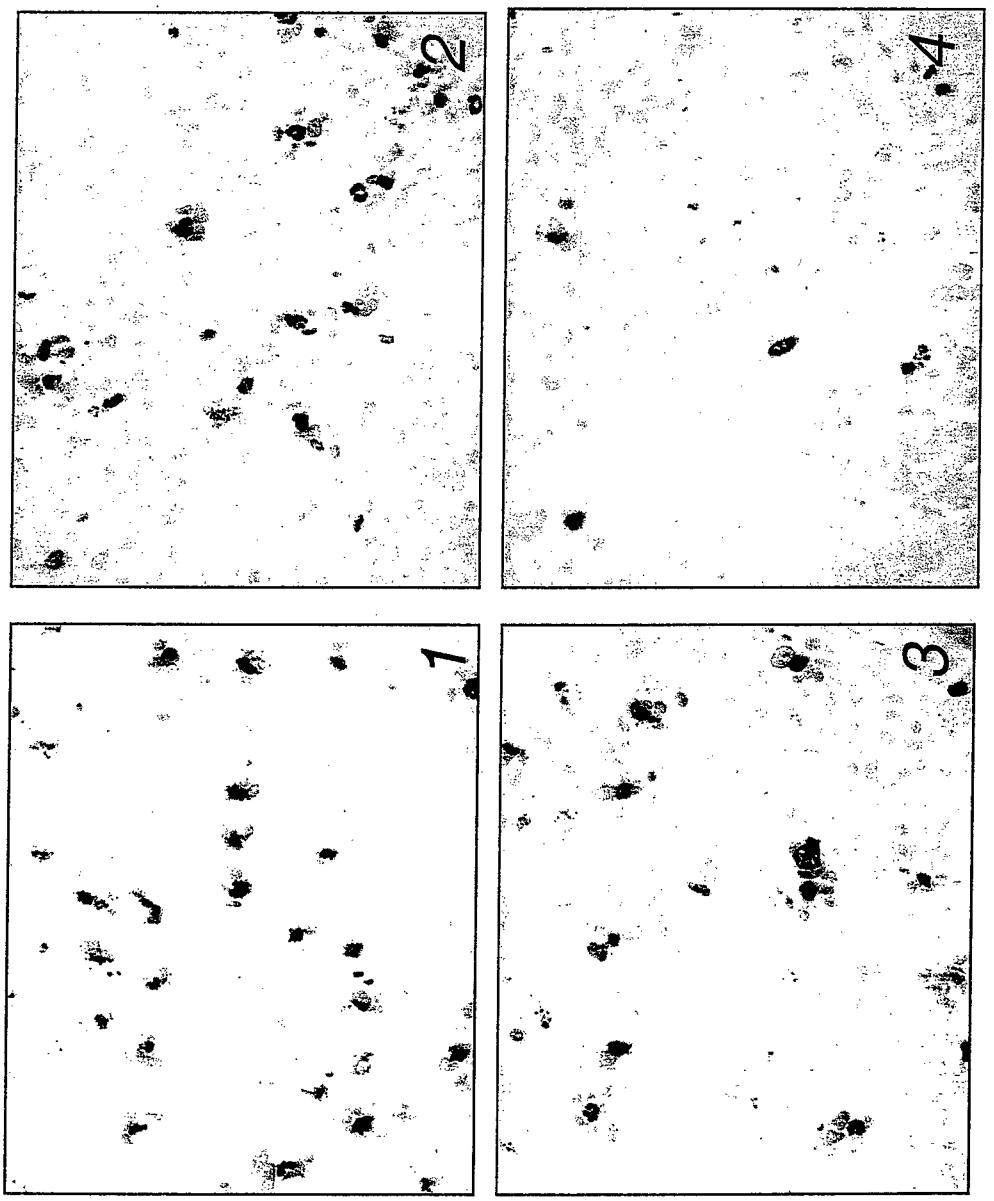


Figure 5B

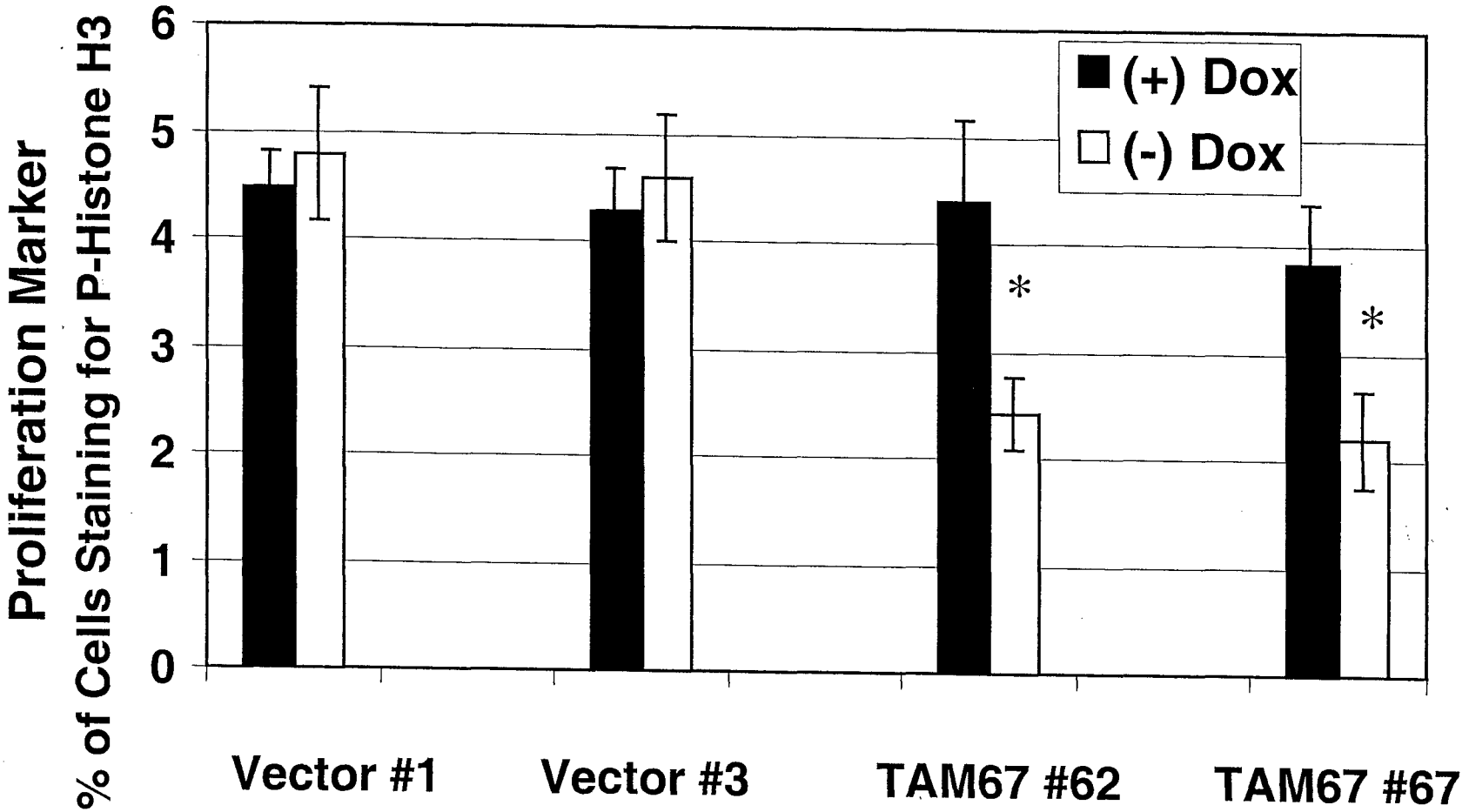
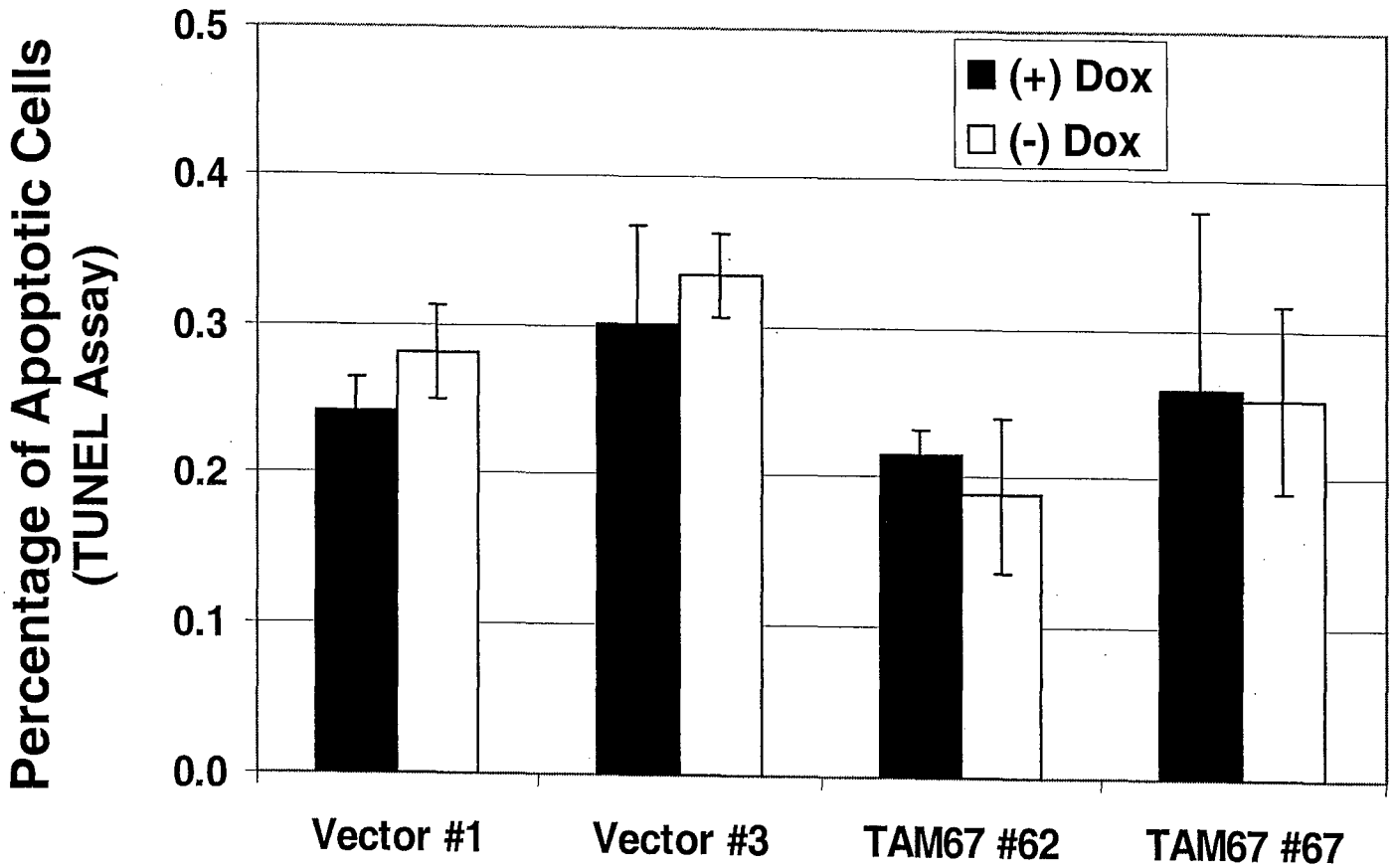

Proliferation Marker
% of Cells Staining for P-Histone H3
0
1
2
3
4
5
6
(+) Dox
(-) Dox
*
*
Vector #1
Vector #3
TAM67 #62
TAM67 #67

Figure 5C


Percentage of Apoptotic Cells
(TUNEL Assay)
0.5
0.4
0.3
0.2
0.1
0.0
(+) Dox
(-) Dox
Vector #1
Vector #3
TAM67 #62
TAM67 #67

Growth Factor Pathways
Growth Factors
TKRs
PI3-K
ERK
JNK
p38
P
P
Jun Fos
AP-1 Site
TAM67
Classical ER Pathway
Non-classical ER Pathway
E_2
E_2
CYTOPLASM
NUCLEUS
ER
ER
ERE
ER
Jun Fos
AP-1 Site
TAM67
CELL PROLIFERATION

Figure 6